

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/64, C12P 19/34	A1	(11) International Publication Number: WO 97/42330 (43) International Publication Date: 13 November 1997 (13.11.97)
(21) International Application Number: PCT/US97/07698 (22) International Filing Date: 6 May 1997 (06.05.97) (30) Priority Data: 08/642,045 6 May 1996 (06.05.96) US (71) Applicant: APOLLON, INC. [US/US]; Suite 30, One Great Valley Parkway, Malvern, PA 19335 (US). (72) Inventors: PACHUK, Catherine, J.; 223 Scottsdale Road, B-511, Lansdowne, PA 19050 (US). SAMUEL, Manoj; 1224 West Chester Pike, West Chester, PA 19382 (US). ZURAWSKI, John, A.; P.O. Box 417, Westtown, PA 19395-0417 (US). SATISHCHANDRAN, C.; 605 Shepard Drive, Lansdowne, PA 19446-5678 (US). (74) Agents: ELDERKIN, Dianne, B. et al.; Woodcock Washburn Kurtz Mackiewicz & Norris LLP, 46th floor, One Liberty Place, Philadelphia, PA 19103 (US).		(81) Designated States: AU, CA, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: CHAIN REACTION CLONING (57) Abstract Chain reaction cloning methods and reagents and kits for performing such methods are disclosed. Chain reaction cloning methods provide a convenient one step process that will allow site-specific ligation of DNA molecules by thermostable DNA ligases. A number of applications are disclosed for chain reaction cloning including site-specific ligation of DNA fragments generated by restriction enzyme digestion, DNase digestion, chemical cleavage, enzymatic or chemical synthesis, and PCR amplification.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

CHAIN REACTION CLONING

FIELD OF THE INVENTION

The present invention relates to methods of synthesizing DNA molecules and to reagents and kits for practicing the method.

BACKGROUND OF THE INVENTION

Conventional cloning techniques rely upon T4 DNA ligase catalyzed ligation of DNA molecules with compatible cohesive or blunt termini. Ligation of cohesive termini is enabled by the formation of Watson-Crick base pairs between nucleotides present at the termini of the two molecules to be ligated. Ligation of molecules with blunt termini is less efficient, since base-pairing does not occur between the termini, and blunt end ligations therefore require higher concentrations of DNA and ligase.

There are several problems inherent with the use of T4 DNA ligase as a catalyst for DNA ligation: 1) T4 DNA ligase cannot catalyze the ligation of DNA molecules with incompatible termini. 2) There is a high frequency of intramolecular ligation when two or more species of DNA to be ligated contain compatible cohesive termini. To reduce the background or intramolecular ligation, it is first necessary to remove the 3' phosphate from one or more of the DNA species prior to ligation. 3) It is not possible to directionally ligate two species of DNA that contain compatible termini. 4) Site-specific ligation cannot occur when three or more species of

- 2 -

DNA with compatible termini are present in the reaction.

There is a need for improved methods of DNA ligation. Applications of such improved method include cloning and polymerase chain reaction (PCR) protocols.

5 PCR is a powerful tool for producing multiple copies of DNA molecules. Using PCR, it is possible to amplify DNA sequences to create thousands and millions of identical copies of DNA molecule. PCR is thus used to clone DNA molecules from sources having at least a single copy of the sequence to be
10 cloned.

PCR operates by providing primers, i.e. short single stranded polynucleotides which have sequences that are complementary to sequences of a portion of the nucleic acid molecule to be amplified. When PCR is performed, the primers
15 and the DNA molecule to be amplified are combined and the temperature raised to denature the DNA molecule to be amplified into single stranded molecules. That is the double stranded DNA molecule dissociates into a sense strand and an antisense strand. The temperature is then lowered to promote
20 hybridization of complementary sequences. Multiple copies of two primers are usually provided, one primer hybridizes to the sense strand of the sequence to be amplified and one primer hybridizes to the antisense strand. Using a thermostable polymerase and free nucleotides, a nucleotide molecule
25 complementary to the sense strand is assembled by adding nucleotides to the 3' end of primer that is hybridized to the sense strand. Each free nucleotide added is complementary to the nucleotide on the sequence to be amplified. As the polymerization continues, a single stranded polynucleotide
30 molecule is assembled nucleotide by nucleotide to be complementary to the sense strand of the sequence to be amplified starting from the 3' end of the primer and proceeding in the direction 5' to 3'. Simultaneously, using polymerase and free nucleotides, a nucleotide molecule complementary to
35 the antisense strand is assembled by adding nucleotides to the 3' end of primer that is hybridized to the antisense strand. Each free nucleotide added is complementary to the nucleotide

- 3 -

on the sequence to be amplified. As the polymerization continues, a single stranded polynucleotide molecule is assembled nucleotide by nucleotide to be complementary to the antisense strand of the sequence to be amplified starting from the 3' end of the primer and proceeding in the direction 5' to 3'. The temperature is then raised to dissociate hybridized complementary sequences after which the temperature is again lowered to promote hybridization. The primers hybridize to the original DNA molecule as well as to the molecules synthesized in the original polymerization. Once hybridized, the polymerase assembles the primers and free nucleotides into a DNA molecule which has a full length complementary sequence to the molecule that the primer is hybridized to. After numerous rounds of lowering temperature, hybridizing primers to molecules, formation of sequences complementary to the molecules by polymerization, raising the temperature to dissociate hybridized and repeating the hybridization/polymerization cycles, most of the amplification products are molecules with sequences identical to the sequence of the original molecule between the two primers.

One shortcoming of PCR is that there is a limit to how long a sequence can be amplified using the technology. If a sequence is greater than the limit for PCR can effectively be used for amplification, it must be amplified as a series of PCR products representing adjacent portion of the final desired molecules. The series of PCR products are ligated together to form the final desired molecules.

There is a need for compositions and methods for amplifying DNA molecules that have sequences which exceed the limit beyond which PCR is effective. There is a need for compositions and improved methods for ligating adjacent PCR products. There is a need for compositions and improved methods for ligating non-adjacent PCR products into one contiguous molecule.

SUMMARY OF THE INVENTION

The present invention relates to chain reaction

- 4 -

cloning (CRC) and to reagents and kits for performing chain reaction cloning methods.

The present invention relates to a convenient one step process that will allow site-specific ligation of DNA molecules with compatible termini in a product-driven reaction. This method utilizes the amplification capability of CRC catalyzed by thermostable DNA ligases. In addition, the method will catalyze the ligation of DNA molecules containing incompatible termini in both product and non-product driven reactions.

The present invention can be used in a number of applications such as, for example: the site specific ligation of DNA fragments generated by restriction enzyme digestion, DNase digestion, chemical cleavage, enzymatic or chemical synthesis. An example of enzymatic synthesis would be PCR synthesis of DNA.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows ligation of two smaller double stranded molecules into a larger double stranded molecule according to the invention using a single bridging oligonucleotide as an initial template.

Figure 2 shows ligation of two smaller double stranded molecules into a larger double stranded molecule according to the invention using two bridging oligonucleotide as initial templates.

Figure 3 is a diagram of PCR amplification of a sequences of a DNA molecule to produce PCR amplification products.

Figure 4 is a diagram of the chain reaction cloning technique of the present invention using the PCR amplification products.

Figures 5A and 5B depict examples of product driven reactions.

Figure 6 is an example of a non-product driven reaction.

Figures 7A, 7B and 7C show site directed mutagenesis

- 5 -

using methods of the present invention.

Figure 8 shows ordered ligation using methods of the present invention.

Figures 9A, 9B and 9C show a long range PCR ligation using a non-strand displacing heat stable DNA polymerase and heat stable ligase.

Figures 10A and 10B show the strategy used to construct the chimeric kanamycin resistance gene described in Example 1. Arrows indicate PCR primers or CRC bridge oligomers, with their MPV numbers (Table 1) indicated above or below. Figure 10A shows the PCR strategy to amplify individual fragments from the indicated templates. The promoter fragment encompasses the promoter and the 5' untranslated region of the *aph(3')*-Ia gene present in pUC4K, including the Shine-Dalgarno sequence. The coding region fragments are derived from the *ant(4')*-Ia gene in pUB110; primer MPV40 alters the Eco47III site. The terminator fragment is also derived from the *aph(3')*-Ia gene in pUC4K. Figure 10B shows the CRC strategy to link the four PCR fragments as described in Example 1. After CRC was performed, some of the sample was amplified by PCR with MPV37 and MPV44.

Figure 11 shows the sequence of the translation initiation region of the engineered *ant(4')*-Ia gene. The vertical line indicates the junction generated by CRC between the promoter and coding region. The Shine-Dalgarno box is underlined. Two reading frames are shown: the upper reading frame represents the desired sequence of the *ant(4')*-Ia gene but begins with GTG, while the lower begins with ATG but is out of frame and terminates quickly (asterisk).

Figures 12A and 12B show the strategy to reconstruct the *ant(4')*-Ia gene as described in Example 1. Figure 12A shows the PCR amplification of fragments from pGEMkm^{ant}. Primer MPV62 incorporates the base changes required to alter the first two codons. Figure 12B shows the CRC strategy to link the two PCR fragments. After CRC was performed, some of the sample was subjected to PCR with MPV64 and MPV63.

Figure 13 shows the DNA sequence of the chimeric

- 6 -

kanamycin resistance gene (SEQ ID NO:1) generated according to Example 1. The initiation and stop codons are underlined and positions of the MPV primers are indicated.

Figure 14 shows construction of the plasmid 23 as described in Example 1. As detailed in the text, the *aph(3')*-*Ia* gene of the starting plasmid 4 was replaced with the chimeric *ant(4')*-*Ia* chimeric gene from pBLUEkm^{ant}. The β -lactamase gene remnant in the plasmid 4 is between the *aph(3')*-*Ia* gene and the BspHI site in the origin.

Figures 15A and 15B show expression of the HSV gene HSVgD₂ in cells transfected with the plasmid 24 as described in Example 1. Figure 15A shows schematic diagrams of two plasmids: plasmid 19 and plasmid 24. Figure 15B shows results from Western blots of RD cells transfected with plasmid 24 (lanes 2,3), plasmid 23 (lanes 4,5) and plasmid 19 (lanes 6,7) as described in Example 1. Lane 1 contains protein molecular weight markers, from top to bottom of blot: 175, 83, 62, 47.5, 32.5, 25, 16.5 and 6.5 kd in size.

Figure 16 shows results from experiments described in Example 1 relating to the growth of plasmid 19 and plasmid 24 in fermentation. Cell mass is measured against fermentation time for *E. coli* harboring either vector. FP5 is fermentation process 5.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the term "composite nucleic acid molecule" is meant to refer to a nucleic acid molecule that is synthesized by ligating at least two separate nucleic acid molecules. A composite nucleic acid molecule is also referred to herein as a "larger DNA molecule" when referring to the product of ligation of two smaller DNA molecules.

The "melting temperature" or "T_m" is calculated as follows:

$$T_m = (16.6 \times \log C) + 81.5 + (0.41 \times \%GC) - (675/L)$$

C = Concentration in molarity of the positive ions.
(where C<0.5M)
L = Length of the oligo (where L<100)
%GC = %GC of matched (base paired) nucleotides

- 7 -

As used herein, the term "denaturing" is meant to refer to the dissociation of a double stranded DNA molecule into single stranded molecules including the dissociation of hybridized nucleic acid molecules of unequal length into single stranded molecules. In addition, "denaturing" means partial dissociation of double stranded DNA such that the termini of the double stranded DNA are available for hybridization to the bridging oligonucleotide. A preferred denaturing temperature is at least the T_m of the oligonucleotide set plus up to 5°C ($T_m + 5^\circ\text{C}$). For most oligonucleotide sets, the denaturing temperature range is $55-98^\circ\text{C}$.

As used herein, the term "annealing" is meant to refer to the hybridization of single stranded molecules with complementary sequences including the hybridization of nucleic acid molecules of unequal lengths. A preferred annealing temperature is the lowest T_m of the oligonucleotide set minus $0-5^\circ\text{C}$ ($T_m - 5^\circ\text{C}$). For most oligonucleotide sets, the annealing temperature range is $40-70^\circ\text{C}$.

The present invention uses a thermostable ligase to ligate single stranded molecules together in a series of ligation reactions which occur in a cycle of temperature changes alternating between annealing temperatures and denaturing temperatures such that DNA molecules present in the reaction mixture alternate between existing as hybridized DNA molecules made up of single stranded molecules hybridized to each other at complementary sequences, and existing as single stranded molecules or as partial single stranded molecules. According to the present invention, nucleic acid molecules are assembled from smaller nucleic acid molecules by a series of ligation reactions. Using a thermostable ligase, nucleic acid molecules can be specifically linked to each other as single stranded molecules without the need for modifying the end of one of the two molecules being joined. The present invention uses denaturing temperature to convert double stranded nucleic acid molecules into single stranded molecules. The ends of the single stranded molecules are brought together by hybridizing

- 8 -

to a template which is either provided as part of the reaction reagents or, after completed cycles, formed by earlier ligation reactions. The use of the template ensures that two single stranded nucleic acid molecules which are intended to be linked
5 are aligned in proximity to each other in correct orientation to allow for the proper 3' end to be adjacent to the 5' end of the molecule to which it is to be ligated. Once oriented by the template, the ligase catalyzes the reaction which covalently joins the two nucleic acid molecules into a single,
10 larger nucleic acid molecule that includes a sequence complementary to the template. The nucleic acid molecules are subsequently subject to denaturing conditions in which the double stranded molecule formed by the ligated nucleic acid molecules and the template cease to hybridize to each other and
15 become single stranded molecules. The conditions are then changed to again favor hybridization of complementary nucleotide sequences. In product driven reactions, in this annealing step, the ligated molecule becomes a template for orienting unligated, single stranded nucleic acid molecules
20 with nucleotide sequences complementary to the nucleotide sequences of the ligated molecule. Accordingly, after a series of alternating denaturing and annealing/ligation steps, nucleic acid molecules are generated from small nucleic acid molecules.

The present invention thus provides the means to
25 generate larger nucleic acid molecules from smaller ones without the need to treat the ends in such a way to ensure specific binding of two molecules in correct orientation. Rather, the template ensures correct orientation and specific ligation. The present invention allows for the ligation of
30 multiple components without the need for intervening steps. Using a thermocycler, a heat stable ligase and the proper starting materials, the series of reactions can proceed with out intervention.

As an initial reaction, at least one bridging
35 oligonucleotide is provided as a template. The bridging oligonucleotide has a nucleotide sequence that includes, from its 3' to 5' ends, at least 10 and preferably 10-40 nucleotides,

- 9 -

complementary to equal number of the most 3' nucleotides of the 3' end of a first single stranded nucleic acid molecule and at least 10 and preferably 10-40 nucleotides complementary to equal number of the most 5' nucleotides of the 5' end of a second single stranded nucleic acid molecule. Thus when the first single stranded nucleic acid molecule and second single stranded nucleic acid molecule are combined together with the bridging oligonucleotide under annealing conditions, i.e. conditions under which complementary nucleotide sequences of nucleic acid molecules hybridize, the complementary sequences of the 3' end of the first single stranded molecule and the complementary sequences of the 5' end of the second single stranded nucleic acid molecules hybridize to the bridging oligonucleotide and are oriented adjacent to each other. In this orientation, the two ends are ligated by the thermostable ligase forming from the two smaller single stranded molecules a first larger single stranded molecule. The bridging oligonucleotide serves as a template to bring together the two smaller single stranded molecules in proper orientation to be ligated.

In some embodiments, a single bridging oligonucleotide is provided as a template for a single ligation reaction between single stranded nucleic acid molecules which are the sense or antisense strands of two nucleic acid molecules to be ligated. Upon restoration to the denaturing conditions, the bridging oligonucleotide is no longer hybridized to the ligated molecule. The bridging oligonucleotide serves as a template for the single ligation reaction between single stranded nucleic acid molecules which are the sense or antisense strands of two nucleic acid molecules to be ligated. The ligated molecule serves as a template for the ligation reaction between single stranded nucleic acid molecules which are the other of the sense or antisense strands of two nucleic acid molecules to be ligated. Thus, in the first round, a single ligation reaction is produced. In the second round, two reactions are produced.

In some embodiments, two bridging oligonucleotides

- 10 -

are provided as templates for two ligation reactions, one between single stranded nucleic acid molecules that are the sense strands of two nucleic acid molecules to be joined and the other one between single stranded nucleic acid molecules that are the antisense strands of two nucleic acid molecules to be ligated. Upon restoration to the denaturing conditions, the bridging oligonucleotides are no longer hybridized to the ligated molecules. The bridging oligonucleotides serve as templates for the two ligation reactions. Similarly, the ligated molecules also serve as templates for single stranded molecules with complementary sequences. Thus, in the first round, two ligation reactions are produced with two templates. In the second round, two reactions are produced with four templates.

15 In some embodiments, CRC may be used to produce large circular molecules from smaller molecules. In such embodiments, a bridging oligonucleotide is provided which circularizes the molecule by ligating the 5' end to the 3' end of a single molecule.

20 The ability to site-specifically ligate two or more DNA molecules containing compatible or non-compatible termini simplifies DNA cloning. Clonings involving the ligation of more than two DNA molecules can be done in one step. Although ligation of more than two DNA molecules can be accomplished by T4 DNA ligase, the efficiency is poor and the screening procedure for isolating the correct ligation product is arduous.

For example, a singular fragment will be cloned into a vector in two orientations using T4 ligase. Directional ligation can be partially achieved by the use of fragments that contain two non-compatible ends. CRC ensures directionality in cloning because only those fragments that hybridize correctly to the oligo can be ligated. An example is given assuming a large number of oligos with compatible termini are involved. When ligating multiple fragments with T4 ligase, the number of ligation products are numerous, and usually greater than a unit length. A unit length is defined as a ligation

- 11 -

product that has each fragment represented once. However, this is not generally the case. Assuming the ligation is stopped following the formation of a unit length, the number of recombinants generated is represented by the formula $2(n^n)$, in which only one is the correctly ligated molecule (In the formula, n = number of fragments). Using CRC to ligate these fragments ensures the formation of unit lengths and the number of such recombinants theoretically equal one per ligated molecule, thus increasing the chances of scoring for the correct ligation product by orders of magnitude. Screening CRC products is simplified since the correct product is always obtained as CRC precludes ligation of termini that are not brought into close proximity by a bridge oligonucleotide.

Thermostable ligase, also referred to as "DNA LIGASE heat-stable, may be obtained from Epicentre Technologies, (Madison, WI). The concentration of thermostable ligase in a reaction is preferably 1-50 units/100ul reaction. In some embodiments, 5 units per 100ul reaction is used.

The amount of bridging oligonucleotide provided in a reaction mixture ranges from 1000 fold less to 1000 fold more relative to the amount of DNA present for ligation. In some preferred embodiments, the ratio is 1 to 1.

The amount of starting material provided in the reaction mixtures ranges from .1 ng to 100 ug DNA.

In some embodiments, the entire CRC reaction can be done in a single cycle.

The number of cycles of alternating denaturing and annealing temperature is usually 5 to 50, preferably 30.

Figure 1 shows the ligation by the methods of the invention using a single bridging oligonucleotide 3 whereby two smaller double stranded molecules $ds1$ and $ds2$ form a larger double stranded molecule $ds5$. The smaller double stranded molecules $ds1$ and $ds2$ are made up of single stranded molecules $1s$ and $1a$, and $2s$ and $2a$, respectively. The molecules exist as double stranded molecules under annealing temperatures. When the temperature is elevated to denaturing temperatures, the molecules exist as single stranded molecules $1s$, $1a$, $2s$ and

- 12 -

2a. In the presence of a bridging oligonucleotide 3 which has a sequence complementary to the 3' most bases of single stranded molecule 1s and the 5' most bases of single stranded molecule 2s and a thermostable ligase 4, the temperature is lowered to annealing temperature and molecules with complementary sequences hybridize. Some of the single stranded molecules 1s, 1a, 2s and 2a reform double stranded molecules ds1 and ds2 and additionally some copies of single stranded molecules 1s and 2s hybridize to bridging oligonucleotide 3.

10 The thermostable ligase 4 ligates the 3' end of single stranded molecule 1s to the 5' end of the single stranded molecule 2s to form a larger single stranded molecule 5s which is the sense strand of the larger double stranded molecule ds5. The temperature is elevated to denaturing temperature and the

15 molecules exist as single stranded molecules 1s, 1a, 2s, 2a, 5s and 3. The temperature is again lowered to annealing temperature and molecules with complementary sequences hybridize. Some of the single stranded molecules 1s, 1a, 2s and 2a again reform double stranded molecules ds1 and ds2 and

20 additionally some copies of single stranded molecules 1s and 2s hybridize to bridging oligonucleotide 3. In addition, single stranded molecules 1a and 2a hybridize to single stranded molecule 5s. The thermostable ligase 4 ligates the 3' end of single stranded molecule 1s to the 5' end of the

25 single stranded molecule 2s to form a larger single stranded molecule 5s which is the sense strand of the larger double stranded molecule ds5 and also ligates the 3' end of single stranded molecule 2a to the 5' end of the single stranded molecule 1a to form a larger single stranded molecule 5a which

30 is the antisense strand of the larger double stranded molecule ds5. The temperature is again elevated to denaturing temperature and the molecules exist as single stranded molecules 1s, 1a, 2s, 2a, 5s, 5a and 3. The temperature is again lowered to annealing temperature and molecules with

35 complementary sequences hybridize. Some of the single stranded molecules 1s, 1a, 2s and 2a again reform double stranded molecules ds1 and ds2 and some copies of single stranded

- 13 -

molecules 5s and 5a form double stranded molecule ds5. In addition, some copies of single stranded molecules 1s and 2s hybridize to bridging oligonucleotide 3, some copies of single stranded molecules 1a and 2a hybridize to single stranded molecule 5s and some copies of single stranded molecules 1s and 2s hybridize to single stranded molecule 5a. The thermostable ligase 4 ligates the 3' end of single stranded molecule 1s to the 5' end of the single stranded molecule 2s to form a larger single stranded molecule 5s which is the sense strand of the larger double stranded molecule ds5 and also ligates the 3' end of single stranded molecule 2a to the 5' end of the single stranded molecule 1a to form a larger single stranded molecule 5a which is the antisense strand of the larger double stranded molecule ds5. The temperature is again elevated to denaturing temperature and the molecules exist as single stranded molecules 1s, 1a, 2s, 2a, 5s, 5a and 3. By alternately cycling of temperature between annealing temperature and denaturing temperature, the larger single stranded molecules 5s and 5a serve as templates for bringing together single stranded molecules 1a and 2a, and 1s and 2s, respectively, in proper orientation, alignment and proximity to be ligated by the thermostable ligase.

Figure 2 shows a similar reaction to that shown in Figure 1 but using two bridging oligonucleotides instead of one. By using the second bridging oligonucleotide 3* which has a sequence complementary to a portion of the sequence of single stranded molecules 1a and 2a, the larger single stranded molecule 5a is formed at an earlier cycle. The formation of the larger double stranded molecule ds5 is not changed.

Multiple ligations may be performed simultaneously. It is contemplated and intended that two, three, four, five etc. smaller molecules may be ligated together in order to form a single larger molecule. Those having ordinary skill in the art can readily adapt the description for ligating two smaller molecules to a single larger molecule to design protocols whereby multiple smaller molecules are joined.

In one preferred embodiment, the present invention

- 14 -

is used in conjunction with multiple simultaneous PCR reactions or that produce multiple PCR products which are then ligated together to form a larger molecule. This embodiment of invention may be described in general terms using the
5 accompanying figures.

Figure 3 shows a PCR reaction which generates multiple PCR amplification products which are ligated together using the present invention in order to form a composite nucleic acid molecule that includes the entire sequence
10 spanning the most 5' and the most 3' primers used. According to Figure 3, a sequence 11 of a nucleic acid molecule 12 is amplified by PCR using four sets of primers 13 and 13', 14 and 14', 15 and 15', 16 and 16'. The sequence 11 consists of a sense strand 11s and an antisense strand 11a. Primers 13, 14,
15 15 and 16 each hybridize to a sequence on the sense strand 11s of the sequence 11. Primers 13', 14', 15' and 16' each hybridize to a sequence on the antisense strand 11a of the sequence 11. When PCR is performed on the nucleic acid molecule 12 using the four sets of primers 13 and 13', 14 and
20 14', 15 and 15', 16 and 16', eight single stranded molecules are formed 13s, 13'a, 14s, 14'a, 15s, 15'a, 16s, 16'a which when complementary strands are annealed form four amplification products 17, 18, 19, 20.

Figure 4 shows the ligation of the four amplification
25 products 17, 18, 19, 20 to produce a DNA molecule with a sequence identical to sequence 11 of nucleic acid molecule 12. The temperature of the four amplification products 17, 18, 19, 20 is elevated to promote dissociation of the double stranded molecules, producing eight single stranded molecules 13s, 13'a,
30 14s, 14'a, 15s, 15'a, 16s, 16'a. Six bridging oligonucleotides 21, 22, 23, 24, 25, 26 are used as templates. Bridging oligonucleotide 21 has a sequence which is complementary to the 3' end of the single stranded molecule 13s and the 5' end of the single stranded molecule 14s. Bridging oligonucleotide 22
35 has a sequence which is complementary to the 5' end of the single stranded molecule 13a and the 3' end of the single stranded molecule 14a. Bridging oligonucleotide 23 has a

- 15 -

sequence which is complementary to the 3' end of the single stranded molecule 14s and the 5' end of the single stranded molecule 15s. Bridging oligonucleotide 24 has a sequence which is complementary to the 5' end of the single stranded molecule 14a and the 3' end of the single stranded molecule 15a. Bridging oligonucleotide 25 has a sequence which is complementary to the 3' end of the single stranded molecule 15s and the 5' end of the single stranded molecule 16s. Bridging oligonucleotide 26 has a sequence which is complementary to the 5' end of the single stranded molecule 15a and the 3' end of the single stranded molecule 16a.

The eight single stranded molecules 13s, 13'a, 14s, 14'a, 15s, 15'a, 16s, 16'a are combined with the six bridging oligonucleotides 21, 22, 23, 24, 25, 26 and the temperature is lowered to promote annealing of complementary nucleotide sequences. Under such conditions, single stranded molecules can hybridize to bridging oligonucleotides. Single stranded molecules 13s and 14s can hybridize to bridging oligonucleotide 21 to form complex 27. Single stranded molecules 13'a and 14'a can hybridize to bridging oligonucleotide 22 to form complex 28. Single stranded molecules 14s and 15s can hybridize to bridging oligonucleotide 23 to form complex 29. Single stranded molecules 14'a and 15'a can hybridize to bridging oligonucleotide 24 to form complex 30. Single stranded molecules 15s and 16s can hybridize to bridging oligonucleotide 25 to form complex 31. Single stranded molecules 15'a and 16'a can hybridize to bridging oligonucleotide 26 to form complex 32. It is possible that complexes can include more than two single stranded molecules and more than one bridging oligonucleotides such as complexes formed by single stranded molecules 13s, 14s and 15s can hybridize to bridging oligonucleotides 21 and 23 to form complex 33. Single stranded molecules 13'a, 14'a and 15'a can hybridize to bridging oligonucleotides 22 and 24 to form complex 34. Single stranded molecules 14s, 15s and 16s can hybridize to bridging oligonucleotides 23 and 25 to form complex 35. Single stranded molecules 14'a, 15'a and 16'a can hybridize to bridging

- 16 -

oligonucleotides 24 and 26 to form complex 36. Complexes may form from four sense or four antisense single stranded molecules. For example, complexes may be formed by single stranded molecules 13s, 14s, 15s and 16s hybridizing to
5 bridging oligonucleotides 21, 23 and 25 to form complex 37. Similarly, single stranded molecules 13'a, 14'a, 15'a and 16'a can hybridize to bridging oligonucleotides 22, 24 and 26 to form complex 38. Once the complexes are formed, the thermostable ligase 4 ligates adjacent nucleotides of single
10 stranded molecules. Upon raising the temperature to a level sufficient for dissociation of double stranded DNA, the bridging oligonucleotide dissociates from the ligated single stranded molecules. Lowering the temperature to annealing temperature brings about the same complex formation as
15 described in the annealing step above and additionally complexes formed using newly formed single stranded molecules 27-38 as templates for joining two, three and four smaller single stranded molecules into one larger single stranded molecules. After multiple denaturing/annealing cycles multiple
20 copies of both the sense and antisense strands of 11 are formed which under annealing conditions provides multiple copies of a double stranded molecule consisting of 11 formed from PCR amplification products 17, 18, 19 and 20. In some embodiments, both sense and antisense primers are used. In some
25 embodiments, only sense primers are used. In some embodiments, only antisense primers are used.

Thus, the present invention may be used to form single double stranded molecules from multiple adjacent PCR products thereby effectively allowing for the PCR amplification
30 of very large nucleotide sequences through the amplification of adjacent sequences and ligation of such products. Alternatively, the present invention may be used to form single double stranded molecules from multiple non-adjacent PCR products thereby effectively allowing for the PCR amplification
35 of very large nucleotide sequences through the amplification of adjacent sequences and ligation of such non-adjacent sequences in a single product.

- 17 -

In Figures 5A, 5B, 6 and 8 only relevant annealings are shown. That is, for the purpose of brevity, only annealings that result in new product formation are shown. For example, in Figure 5A, in cycle 1, $n > 3$, A can reanneal to A' and B can reanneal to B' and no new product is generated. These annealings are not favored at any point in the reaction as there is a molar excess of o' with respect to A and B. Therefore most of A and B will anneal to o'. In addition, some subset of o' will hybridize only to A or B. In cycle 2 $N > 3$, AB can anneal to o' and no new product is generated. In cycle 3, $n > 3$, AB and A'B' can reanneal to each other and no new product is generated.

Figures 5A and 5B are examples of product driven reactions. Figure 5A depicts DNA fragments containing compatible termini and their participation in a product driven reaction. The picture example is of two blunt ended DNA molecules. Other types of termini that can be ligated to each other in a product-driven reaction are a 3' overhang to a blunt terminus and a 5' overhang to a blunt terminus and also a 5' overhang and a 3' overhang when a heat stable non-strand displacement DNA polymerase (such as: Amplitherm DNA polymerase, Epicentre Technologies Madison WI; Tfl DNA polymerase, Epicentre Technologies Madison WI, Promega Madison WI; Tth DNA polymerase, Epicentre Technologies Madison WI, Promega Madison WI; Replitherm DNA polymerase, Epicentre Technologies Madison WI; Pfu DNA polymerase, Stragene LaJolla CA; and Exo-Pfu DNA polymerase, Stragene LaJolla CA) is included together with heat stable DNA ligase (Ampligase, Epicentre Technologies, Madison WI) in the reaction as shown in Figure 5B. Molecules containing 5' overhangs only or 3' overhangs only must first be blunt ended prior to ligation. Ligation of blunt ended molecules occurs in a product driven reaction.

In Figure 5A, the top strand (A) and the bottom strand (A') of DNA molecule 1, the top strand (B) and the bottom strand (B') of DNA molecule 2 are depicted. The bridge oligonucleotide is designated 0. Base pairing is indicated

- 18 -

with vertical lines. During cycle 1, 0, which is complementary to the terminal sequences of A and B, anneals to strands A and B. Ligation of strands A and B occur, resulting in the formation of product molecule AB. Ligation is represented by the filled circle (•). In cycle 2, product AB anneals to strands A' and B', ligation of A' and B' occur, and product A'B' is formed. In cycle 3, A'B' acts as a catalyst for the formation of product AB. In cycle $n > 3$, molecules A'B' and 0 act as catalysts for the formation of product AB while product AB acts as a catalyst for the formation of product A'B'.

In Figure 5B, the top strand (A) and the bottom strand (A') of DNA molecule 1, the top strand (B) and the bottom strand (B') of DNA molecule 2 are depicted. DNA molecule 1 contains a 5' recessed end while DNA molecule 2 contains blunt ends. The bridge oligonucleotide is designated 0. The 3' end of 0 contains a blocking group (*) so that 0 cannot be chain extended. Base pairing is indicated with vertical lines. During cycle 1 and cycles $n > 1$, 0, which is complementary to the 3' terminus of A and the 5' terminus of B, anneals to strands A and B. Ligation of strands A and B occur, resulting in the formation of product molecule 5'-AB-3'. Ligation is represented by the filled circle (•). In cycle 2 and cycles $n > 2$, product 5'-AB-3' anneals to strands A' and B'. In the presence of a non-strand displacing heat stable DNA polymerase, B' is extended until the growing B' chain incorporates the nucleotide immediately 3' of the most 5' nucleotide of A' (that is to say that the template molecule is copied up to the nucleotide located immediately 3' of the first nucleotide that is based paired with the 5' most nucleotide of A'). Ligation of A' and B' occurs, and product 5'-A'B'-3' is formed. In cycle 3 and in cycles $n > 3$, 5'-A'B'-3' acts as a catalyst for the formation of product 5'-AB-3'.

Figure 6 shows an example of a non-product driven reaction (i.e. ligation of non-compatible molecules bearing a 5' overhang and a 3' overhang. An example of this would be ligation a molecule cut with BamH1 to a molecules cut with

- 19 -

Pst1.) In this type of reaction, the product DNA strand does not catalyze the ligation of the other DNA molecules. The picture examples depict the ligation of a DNA molecule with a 5' overhang to a DNA molecule with a 3' overhang. The ligation occurs in a non-product driven reaction in the absence of a heat stable non-strand displacing DNA polymerase. In Figure 6, DNA molecule 1 containing the 5' overhang and DNA molecule 2 containing the 3' overhang are depicted. The Δ and \circ regions of DNA molecules 1 and 2 designate complementary sequences to the corresponding Δ and \circ regions of bridge oligonucleotide 0. During cycle 1, 0 anneals to strands A' and B'. Ligation of strands A' and B' occur, resulting in the formation of product molecule A'B'. In cycle 2, product A'B' anneals to strands A and B. The gap in the molecules can be filled with a DNA polymerase and subsequently used to transform bacteria or used to transform bacteria with no prior fill in. In a non-product driven reaction, CRC may be practiced by holding the reaction at one temperature for a long period of time, following an initial 98°C denaturation. The temperature for holding the reaction will be the optimum for enzyme activity, ~65°C degrees. The bridge oligos would be designed such that the T_m < 65°C, preferably 55-60°C. At this temperature, the oligos will hybridize to target sequences and bring the 2 strands together, but inefficiently since the temperature is above the T_m . Following ligation, the oligo dissociates and is available for another reaction. The total separation of template DNA need not be complete, however the ends of the template DNA need to denature so as to allow the hybridization of the bridge oligo. At the end of the reaction, the reaction is heated to 98°C and slowly cooled to allow annealing of the DNA strands.

Figures 7A, 7B and 7C show site directed mutagenesis using methods of the present invention. In Figure 7A, o' is complimentary to the entire region to be deleted. As this region becomes larger, the length of o' also becomes larger and more expensive. If the cost of o' becomes prohibitive, the experimenter may opt to perform the experiment according to an

- 20 -

alternative method detailed in Figure 7C. The source of ss DNA for site-directed mutagenesis can be ss phage DNA or denatured plasmid DNA.

Figure 7A depicts site directed deletion of sequences. The parental DNA is depicted as a single stranded circular DNA. The sequence to be deleted from the parental DNA, represented by the filled rectangle (\blacksquare), is annealed to a complementary oligonucleotide, (o'), containing a 3' blocking group. The 3' block, indicated by an asterisk (*), prevents oligonucleotide extension by polymerases. Oligonucleotide 2 (o^2) is complementary to nucleotide located immediately 3' of the sequence to be deleted. o^2 is extended in the presence of a non-strand displacing DNA polymerase such as T4 DNA polymerase and dNTPs. The ends of the newly synthesized strands (represented by \circ and Δ) are annealed to the bridge oligonucleotide o^3 and ligated, resulting in the deleted product DNA molecule.

Figure 7B depicts site directed insertion of sequences. The parental DNA is depicted as a single stranded circular DNA. The sequence to be inserted is shown as the non-annealed portion of o' . The 5' terminus of o' is represented with a Δ . Immediately flanking the 3' end of the sequence to be inserted is a sequence that is complementary to the parental DNA. o' is extended in the presence of T4 DNA polymerase and dNTPs. The ends of the newly synthesized strands (represented by \circ and Δ) are annealed to the bridge oligonucleotide o^2 , and ligated, resulting in the DNA produced containing an insertion (\square).

In Figure 7C, the parental DNA is depicted as a single stranded circular DNA. The sequence to be deleted from the parental DNA is represented by the filled rectangle (\blacksquare). o' , complementary to the region immediately upstream of the region to be deleted, carries a 3' blocking group indicated by an asterisk (*). The 3' block prevents oligonucleotide extension. o^2 is complementary to nucleotide located immediately downstream of the sequence to be deleted and is extended in the presence of a non-strand displacing DNA

- 21 -

polymerase such as T4 DNA polymerase and dNTPs. The ends of the newly synthesized strands (represented by \square and Δ) are annealed to the bridge oligonucleotide o^3 . The bridging oligonucleotide is comprised of the sequence that is complementary to the entire O' and is flanked on the 3' side by the complement to the \square sequence and on the 5' side to the Δ sequence. Following the fill in reaction, both strands contain the O' sequence and have sustained the desired deletion.

10 An ordered ligation is depicted in Figure 8. DNA molecules 1,2 and 3 are shown. All termini of these molecules are compatible. In order to specifically ligate the Δ terminus to the \square terminus and the o terminus to the $\square\square$ termini, the mixture of DNA molecules is annealed to the two bridging
15 oligonucleotides, o' and o^2 . Product molecules $A'B'$, $B'C'$ and $A'B'C'$ are generated which in subsequent cycles act to catalyze the formation of products AB BC and ABC.

Figures 9A, 9B and 9C show a PCR ligation. In Figure 9A, PCR primers $p1$, $p2$, $p1'$ and $p2'$ are shown annealed to the
20 DNA templates. The primers are depicted by short lines while the single stranded DNA templates are depicted by the longer lines. Figure 9B shows a PCR reaction that contains both a non-strand displacing heat stable DNA polymerase and a heat stable ligase, the PCR primers are chain extended. The
25 direction of the extension is indicated by arrows. As shown in Figure 9C, when the extending 3' terminus of the primer extension products $p1$ extend and $p1'$ extend, abut the 5' terminus of the downstream primer extension products, $p2$ extend and $p2'$ extend, ligation occurs resulting in covalent
30 attachment of adjacent DNA molecules. Ligation is indicated by the filled in circle (\bullet). PCR ligation is especially useful to make large PCR products with extension times that are short and reasonable and do not compromise enzymatic activity during the course of PCR. The extension times required in a PCR
35 ligation method is determined by the distances between the primers $p1$ and $p2$.

CRC can also be used to enable long-range PCR. This

- 22 -

can be accomplished by the site-specific ligation of PCR products or by performing PCR in the presence of a non-strand displacing heat stable DNA polymerase and a heat stable ligase as shown in Figures 9A, 9B and 9C.

5 CRC can also be used to specifically clone a specific fragment of DNA from a pool of DNA fragments; for example, cloning a specific DNA fragment following a limited DNase digestion of a DNA molecule as would be done for the creation of a set of nested deletions. Another example is cloning a
10 specific fragment of DNA following restriction enzyme digestion that yields multiple fragments of DNA. In addition, CRC can be used for site directed mutagenesis as shown above. CRC can also be used to circularize a linear piece of DNA by using a bridge oligonucleotide containing complementarity to both
15 termini of the linear DNA.

The present invention provides kits for cloning genes into vectors. According to some embodiments, kits comprise a container having in it a vector such as plasmid, phage, viral vector, yeast artificial chromosome, or other vector into which
20 a desired DNA molecule is to be inserted. In addition, the kits comprise adaptors which are ligated to the ends of a desired DNA molecule when combined with the desired DNA molecule in the presence of ligase. Further the kits comprise bridge oligonucleotides which will hybridize to the ends of the
25 adaptors and the ends of the vector at the insertion point. Additionally, the kits comprise a container having heat stable DNA ligase. Optionally, the kits include DNA ligase for joining the adaptors to the desired DNA molecule.

The present invention provides improved PCR kits
30 which, in addition to including heat stable polymerase, primers dNTPs, and vectors, further comprise bridge oligonucleotides designed to hybridize to primer sequences and vector sequences and heat stable DNA ligase. Using such kits, cDNA libraries may be prepared by PCR. The cDNA clones are inserted into
35 vectors in the correct orientation using bridge oligonucleotides according to the invention.

EXAMPLE

INTRODUCTION

Clinical vectors have been modified to replace the *aph(3')*-Ia gene with a chimeric kanamycin resistance gene. To
5 compare the ability of either backbone to express eukaryotic genes, the envelope glycoprotein D gene (HSVgD₂) from herpes simplex virus 2 (HSV-2) was cloned into clinical vectors which had either one of the two kanamycin resistance genes. In tissue culture experiments, both vectors support expression of
10 HSVgD₂ protein as detected by Western blot. Fermentation parameters of *E. coli* containing either vector were also compared. Growth of cells harboring the chimeric *ant(4')*-Ia gene was considerably enhanced when compared to cells harboring the *aph(3')*-Ia gene, although DNA yields per gram of cell were
15 similar for either vector. The growth differences are most likely a consequence of the different biochemical requirements and activities of ANT(4')-IA enzyme and APH(3')-IA enzyme.

MATERIALS AND METHODS

Plasmids:

20 The kanamycin resistance gene aminoglycoside 3'-phosphotransferase type Ia (*aph(3')*-Ia) was obtained from the plasmid pUC4K (Pharmacia, Piscataway, NJ). This *E. coli* gene for resistance to kanamycin was originally derived from Tn903.

The kanamycin resistance gene adenylyl 4'-
25 nucleotidyltransferase type Ia (*ant(4')*-Ia) (Matsumura et al., *J. Bacteriology* 1984, 160:413-420 which is incorporated herein by reference) was obtained from the plasmid pUB110 (Sigma, St. Louis, MO). The pUB110 plasmid was originally discovered in gram positive *S. aureus*.

30 The clinical DNA vector is a plasmid backbone that contains a bacterial origin of replication, a composite promoter comprised of the Rous sarcoma virus (RSV) enhancer and the human cytomegalovirus (HCMV) immediate early promoter, a polylinker for insertion of a gene encoding a desired protein or antigen, an SV40 polyadenylation signal, and a kanamycin
35 resistance gene. The original plasmid, plasmid 4, contains each of the elements described above and the kanamycin

- 24 -

resistance *aph(3')*-Ia gene.

Plasmid 19 is the plasmid 4 vector with the HSV gene HSVgD₂ cloned between the promoter and polyadenylation signal.

Plasmid 23 is a modification of plasmid 4 in which
5 the *aph(3')*-Ia gene is replaced with the chimeric *ant(4')*-Ia gene of the invention.

Plasmid 24 is plasmid 23 with the HSVgD₂ gene cloned between the promoter and polyadenylation signal.

Bacterial Strains:

10 *E. coli* DH10B (*F*⁻ *mcrA*, Δ (*mrr-hsdRMS-mcrBC*) ϕ 80*dlacZ* Δ M15 Δ *lacX74* *deoR* *recA1* *endA1* *araD139* Δ (*ara,leu*) 7697 *galU* *galK* λ -*rpsL* *nupG*) competent cells (Gibco-BRL, Grand Island, NY) were transformed according to the manufacturer's instructions with plasmid 4, plasmid 19, plasmid 23 and plasmid
15 24, and grown on LB plates containing 40 μ g/ml kanamycin. Plasmid DNA was purified by the alkaline lysis procedure (Sambrook, S., et al., *Molecular Cloning: A Laboratory Manual* 1989, which is incorporated herein by reference). DH10B cells were transformed with pBLUEkm^{ant} and pUC4K, in order to analyze
20 the range of activity of *ant(4')*-Ia and *aph(3')*-Ia genes, respectively, against various aminoglycosides. These experiments were carried out by Microbiology Reference Laboratory, Cypress, CA.

Primers and Bridge Oligomers:

25 DNA oligomers were designed for use in polymerase chain reaction (PCR) or in chain reaction cloning (CRC as described below), and were supplied by Research Genetics, Huntsville, AL. Table 1 lists the primers and oligomers, and Figures 10A, 10B, 12A, 12B and 13 indicate their positions in
30 relation to the templates and the final chimeric *ant(4')*-Ia sequence. PCR primers were stored as 100 μ M stocks in sterile water, while bridge oligomers were stored at 1 mg/ml in sterile water.

PCR Reaction Conditions:

35 Reactions were performed in 50 μ l volumes containing 1X PCR buffer (50 mM KCl, 10mM Tris, pH 8.3, 1.5 mM MgCl₂, 0.001% gelatin), 200 μ M each dNTP, 0.2 μ M each primer, 1 unit

- 25 -

AmpliTag® thermostable polymerase (Perkin-Elmer), and 5 ng of template DNA. Samples went through 30 cycles of 94°C 1 minute, 72°C 1-2 minutes in a Perkin Elmer 9600 machine.

During the first round of cloning, the engineered
5 ant(4')-Ia gene was initially amplified to include FseI and
SwaI sites at the 5' and 3' ends (primers MPV37 and MPV44), for
use in future cloning experiments. When the gene was subjected
to PCR to alter the first and second codons, XbaI and BamHI
sites were additionally engineered onto the 5' and 3' ends of
10 the gene (primers MPV64 and MPV63, respectively), to enable
easy cloning into those same sites in pBluescript.

CRC Reaction Conditions:

Chain reaction cloning (CRC) employs a thermostable
ligase to join DNA fragments in a desired order. It is often
15 difficult to make gene constructs because DNA fragments lack
either compatible restriction enzyme sites, or enzyme sites at
the "right" places. This method obviates the need for such
sites, because it joins fragments in a precise order determined
by the experimenter. One need only know the sequence at the
20 ends of the fragments to be joined. A "bridge" oligomer is
designed which is identical to a desired junction region, and
which overlaps the two fragments to be joined by approximately
20 to 25 bases on each side of the junction. The two fragments
are incubated in equimolar ratios with an excess of the bridge
25 oligo, and heated to 94°C to melt the DNA strands. The sample
is cooled to 68-72°C, enabling the bridge oligo to hybridize
to the single strands from the two fragments. The oligo brings
together these single strands so that the ligase can join them
together. This cycle is repeated many times, and in subsequent
30 cycles both the bridge oligo and previously joined single
strands act as templates for hybridization and ligation. Once
CRC is completed, a portion of the sample is usually subjected
to PCR, using primers derived from the ends of the joined
fragments, and the amplified DNA can be cloned and analyzed.

35 CRC was employed to join four fragments in a specific
order to generate the engineered ant(4')-Ia gene, while two
fragments were joined by CRC to generate plasmid 23.

- 26 -

DNA fragments used in CRC were obtained through PCR or restriction digestion. In either case, the fragments were separated on low-melt agarose gels and purified (Sambrook et al., 1989 *Supra*). Reactions were in 100 μ l volumes containing 5 equimolar amounts of the fragments to be ligated (up to 1 μ g of each fragment), 8-10 picomoles of each bridge oligo, 1X CRC buffer (20 mM Tris, pH 8.3, 25 mM KCl, 10 mM MgCl₂, 0.5 mM NAD, 1% Triton X-100), and 50-100 units of Ampligase® (Epicentre, Madison, WI). Samples went through 50 cycles of 94°C 1 minute, 10 68-72°C 2 minutes. When CRC products were to be resolved and amplified by PCR, approximately 5% to 40% of the CRC reaction was used as template for PCR.

Subcloning, Ligations and Transformations:

Some DNA fragments obtained by PCR amplification were 15 ligated into the plasmid pCR™3, and the ligation products were used to transform *E. coli* one shot™ TOP10F' cells, according to the manufacturer's instructions (Invitrogen, San Diego, CA). The ant(4')-Ia engineered gene was initially cloned this way, to yield plasmid pkm23. The ant(4')-Ia gene was excised from 20 pkm23 with XbaI and BamHI and subcloned into the same sites in pGEM11Zf+ for functional testing, to yield plasmid pGEMkm^{ant}. DNA from pGEMkm^{ant} was the template for the reconstruction of ant(4')-Ia. After the altered gene was generated by PCR and CRC, it was cleaved at engineered XbaI and BamHI ends and 25 subcloned into those sites in pBluescript, yielding pBLUEkm^{ant}.

The HSVgD₂ gene in plasmid 19 was excised from that plasmid with KpnI and MluI. The fragment was ligated into the same sites present in plasmid 23, to yield plasmid 24.

The above conventional ligations were performed in 30 a final volume of 10 to 15 μ l, where the vector to insert molar ratio was approximately 1:3. Vectors were digested with appropriate restriction enzymes, then treated with calf intestinal alkaline phosphatase, as directed by the manufacturer (New England Biolabs, Beverly, MA). Up to 500 ng 35 of vector was ligated to an appropriate amount of insert in 60 mM Tris, pH 7.6, 7 mM MgCl₂, 10 mM DTT, 1 mM ATP, and 400 units of T₄ ligase, and incubated at 14°C overnight. These ligations

- 27 -

were used to transform *E. coli* DH10B cells (Gibco-BRL, Grand Island, NY) according to the manufacturer's protocol.

The ant(4')-Ia gene was ligated into plasmid 4 by CRC (Figure 14). Plasmid 4 was cleaved with DraI and BspHI, and the 2.6 kb fragment generated by these enzymes was gel-purified. The 5' overhang generated by BspHI digestion was blunted with Klenow (Sambrook et al., 1989 *Supra*). The 1.2 kb ant(4')-Ia gene fragment was excised from pBLUEkm^{ant} using NaeI and SmaI, which generate blunt ends, and the fragment was gel-purified. The desired fragments were subjected to CRC with bridge oligomers MPV73 and MPV92, and then the reaction was concentrated by precipitation and resuspended in 10 μ l of TE (10 mM Tris, 7.6, 1 mM EDTA). One μ l of the CRC reaction was used to transform *E. coli* DH10B cells (Gibco-BRL, Grand Island, NY).

DNA Sequencing:

The Sequenase system (USB, Cleveland, OH) was employed for most of the sequencing performed. Approximately 50 ng of any given primer was used to prime a sequencing reaction. If a sequence could not be read by the Sequenase enzyme because of compressions, then the *fmol*[®] DNA sequencing system (Promega, Madison, WI) was used to resolve the discrepancies.

Cell Lines, Transfection Conditions, and Western Blots:

The human rhabdomyosarcoma cell line RD was maintained in MEM, alpha modification (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum, nonessential amino acids and sodium pyruvate. Cells were seeded into six-well plates, and transfected the next day with plasmid 19, plasmid 23, or plasmid 24 by the modified calcium phosphate method (Sambrook et al., 1989 *Supra*), or by lipofectamine according to the manufacturer's instructions (Gibco-BRL, Grand Island, NY).

To determine if HSVgD₂ was produced by the cells, 48 hours after transfection the cells were lysed for Western blotting (Sambrook et al., 1989 *Supra*). Lysates were subjected to SDS-PAGE, and electroblotted to nitrocellulose. The blot

- 28 -

was blocked with 0.5% Tween-20 and 5% nonfat dry milk in TBS, and incubated with the anti-HSVgD₂ monoclonal antibody D1-6 diluted 1:250 in the same buffer. The blot was incubated with a secondary antibody, an anti-mouse IgG polyclonal antibody
5 conjugated to alkaline phosphatase (Jackson ImmunoResearch, Bar Harbor, ME). Binding was then detected by incubation with substrates NBT/BCIP (Promega, Madison, WI).

Fermentations and Plasmid DNA Purification:

Fermentations were performed for *E. coli* DH10B
10 containing either plasmid 19 or plasmid 24. The protocol used was fermentation process 5 (FP5). The growth profiles for either strain were very similar, and thus only one profile for each is shown in Figure 16. Plasmid DNA was purified as described (Gayda 1995).

15 RESULTS AND DISCUSSION

Construction of the ant(4')-Ia Gene by PCR and CRC:

The ant(4')-Ia gene is derived from gram positive organisms. Its promoter, ribosome binding sites, and terminator are optimal for expression in such bacteria, but not
20 for gram negative *E. coli*. The selectivity of gram negative promoters is due to the use of a single sigma factor versus the cascade of sigma factors required in gram positive organisms such as *B. subtilis*. In addition, gram negative bacterial ribosomes require that transcribed RNA contain specific signals
25 for translation, which are lacking in RNA from gram positive organisms.

Initially, the coding region from the ant(4')-Ia gene was linked to the promoter and terminator from the aph(3')-Ia gene, which expresses well in *E. coli*. In addition, an
30 Eco47III site within the ant(4')-Ia gene coding region needed to be eliminated for purposes of future cloning, but only a single base had to be altered, which did not change the protein sequence. PCR was used to individually amplify the aph(3')-Ia promoter, including the ribosome binding site, and the
35 terminator sequences. The ant(4')-Ia gene coding region was likewise amplified in two pieces, with the antisense primer of the 5' fragment altering the Eco47III site.

- 29 -

The fragments were mixed in roughly equimolar amounts, with an excess of bridge oligomers to hybridize and join the fragments in the correct order. The fragments were subjected to CRC (Figure 10B), and approximately 40% of the CRC
5 reaction was then subjected to PCR. This second PCR reaction employed the two outermost primers, MPV37 and MPV44, which amplified across the entire length of the engineered gene. The PCR products were ligated into the pCRTM3 vector, transformed into *E. coli*, and selected on LB ampicillin plates.

10 Of fifty clones selected for analysis, three were full length representations of the engineered *ant(4')-Ia* gene. One clone (pkm23) was fully sequenced, and found to be identical to the various input DNAs and with the correct junctions between each PCR fragment. This clone was selected
15 for functional analysis.

The pCRTM3 vector already contained a kanamycin resistance gene, so it was not possible to determine directly if *ant(4')-Ia* gene were functional in pkm23. The *ant(4')-Ia* gene insert of pkm23 was subcloned into pGEM11Zf+, a vector
20 which only contains an ampicillin resistance gene. While the subcloning was successful, the bacteria containing pGEMkm^{ant} plasmid grew only on plates containing ampicillin, not on plates containing kanamycin. Thus, the engineered *ant(4')-Ia* gene was not functional.

25 Reconstruction of the *ant(4')-Ia* Gene:

Closer examination of the translation initiation region of the engineered *ant(4')-Ia* gene suggested that it was not functional because it was not translated correctly in *E. coli*. Translation initiation regions in *E. coli* genes are
30 characterized by a purine-rich ribosome binding sequence, called the Shine-Dalgarno box, followed 5 to 15 bases downstream by the translation initiation codon, usually the first ATG of the coding sequence. One of the many differences between gram negative and gram positive organisms is that the
35 former almost always use ATG as the start codon, but the latter use ATG or GTG. In fact, the GTG codon is poorly recognized as the initiation codon by gram negative bacteria.

- 30 -

The engineered ant(4')-Ia gene contains a Shine-Dalgarno box from the aph(3')-Ia promoter, but it is followed by two potential start codons from the ant(4')-Ia coding sequence: the in-frame GTG and an out-of-frame ATG that are 5 and 9 bases downstream, respectively (Figure 11). Only translation from the GTG would give rise to a functional enzyme, but it is unlikely to be recognized as the start codon by *E. coli* ribosomes.

Based on the above analysis, the translation initiation region was altered, from GTG AAT GGA to ATG AAC GGA. Changing the bold-faced bases does not alter the protein sequence. Again, a combination of PCR and CRC was employed to generate these mutations, as detailed in Figures 12A and 12B. The pGEMkm^{ant} plasmid served as template, in which the promoter was amplified in one reaction, and the coding region and terminator in another reaction. The sense primer used to amplify the coding region and terminator incorporated the desired nucleotide changes. The PCR fragments were then linked by CRC, and the products were amplified by a second round of PCR using the outermost primers to amplify the entire gene. The final PCR product was cleaved at unique sites on the 5' and 3' ends, and cloned directly into pBluescript which only carries an ampicillin resistance gene. The ligations were transformed into *E. coli*, and grown on plates containing kanamycin. Twenty-two colonies were obtained, and three were sequenced in the junction region between the promoter and coding region. All three had the corrected first and second codons. The ant(4')-Ia gene of one of the three clones was then sequenced, and found to be otherwise identical to the pGEMkm^{ant} template (see Figure 13). This clone is designated pBLUEkm^{ant} and it contains an insert of 1200 bp, with an open reading frame of 254 amino acids, flanked by a 5' promoter sequence of 130 bp and a 3' terminator of 308 bp.

Aminoglycoside Sensitivity of *E. coli* Carrying ant(4')-Ia:

A sensitivity/resistance profile to seven of the most frequently prescribed aminoglycosides was determined for *E. coli* carrying either the ant(4')-Ia gene or the aph(3')-Ia

- 31 -

gene. The pBLUEkm^{ant} and pUC4K plasmids were transformed into *E. coli* DH10B, a strain which carries a streptomycin resistance marker. The transformed strains and the host strain were tested against a series of aminoglycosides to determine their minimum inhibitory concentrations (MIC). Results are shown in Table 2, with MICs shown in $\mu\text{g/ml}$, and resistance or sensitivity indicated. All strains are resist to streptomycin as expected, but neither the *ant(4')-Ia* gene nor the *aph(3')-Ia* gene is expected to confer resistance to this antibiotic (Shaw et al., 1993). The *E. coli* strain alone is sensitive to the remaining antibiotics, providing a baseline of comparison for the bacteria carrying the plasmids with the *ant(4')-Ia* gene or the *aph(3')-Ia* gene. The data show that the *ant(4')-Ia* gene confers resistance to kanamycin, neomycin, and tobramycin, while the *aph(3')-Ia* gene confers resistance to kanamycin, neomycin, tobramycin, gentamicin and netilmicin. The most significant difference between the two genes is that the *ant(4')-Ia* gene is sensitive to gentamicin, an antibiotic that is still the first course of treatment for gram negative infections. Thus, the engineered *ant(4')-Ia* gene fulfills the requirement that it display a narrower range of activity against aminoglycosides, and should be safer for use in humans. Replacement of the *aph(3')-Ia* Gene in plasmid 4 with *ant(4')-Ia*:

The *ant(4')-Ia* gene was cloned by CRC into plasmid 4, to replace the *aph(3')-Ia* gene contained in this vector backbone. Plasmid 4 was cleaved with DraI and BspHI, which eliminates the *aph(3')-Ia* gene and a remnant of the β -lactamase gene left in the plasmid during its original construction. The DraI site is at the 3' end of the SV40 polyadenylation signal. Cleavage at this site removes 42 bases at one end of the element, which is not expected to affect its function. The modified clinical vector backbone resulting from this work is designated plasmid 23. Restriction analysis of plasmid 23 and sequencing of the junctions between the plasmid 4 fragment and *ant(4')-Ia* fragment in plasmid 23 verified that the fragments went together in the desired orientation.

- 32 -

In plasmid 4, *aph(3')*-Ia transcription was directed toward the origin. The terminator of *aph(3')*-Ia is rho-dependent, and rho-dependent terminators can allow a low level of readthrough transcription to occur (Darnell, J. et al.,
5 *Molecular Cell Biology*, 1986, which is incorporated herein by reference, and Miller, J.H. et al., *The Operon* 1980 which is incorporated herein by reference), in this case originating from the *aph(3')*-Ia promoter. The readthrough could result in additional RNA II transcription from the origin. Plasmid
10 replication is, in part, a function of the binding of RNA I to RNA II (Kues, U. et al., *Microbiol. Rev.* 1989, 53:491-516, which is incorporated herein by reference), and the extra RNA II transcription might be expected to result in lower plasmid copy number per cell. To get around this potential problem,
15 the *ant(4')*-Ia gene was ligated into plasmid 4 so that its transcription is directed away from the origin.

Expression of HSVgD₂ from plasmid 19 and plasmid 24:

When plasmid 23 was constructed, a small portion of the SV40 polyadenylation signal was deleted as described above.
20 This deletion did not include the AATAAA sequence, or the GT-rich region required for efficient polyadenylation, but it remained possible that this deletion could adversely affect expression of the eukaryotic gene unit. To evaluate this concern, the HSVgD₂ gene from plasmid 19 was cloned into
25 plasmid 23, to yield plasmid 24 (Figure 15A). The only differences between plasmid 19 and plasmid 24 are the polyadenylation signals, and the *aph(3')*-Ia and *ant(4')*-Ia genes, respectively.

Expression studies were performed, in which RD cells
30 were transfected with either plasmid 19, plasmid 23 or plasmid 24. Results are shown in Figure 15B. Cells transfected with either of the vectors containing HSVgD₂ produce substantial amounts of the 55 kilodalton HSVgD₂ protein as detected by Western blot, while the lanes representing the control plasmid
35 are negative. These data suggest that the small deletion in the SV40 polyadenylation signal does not adversely affect eukaryotic gene expression from the vector. In addition, the

- 33 -

presence of the *ant*(4')-Ia gene coding sequence in the vector does not appear to affect expression from the eukaryotic promoter.

Fermentation and Plasmid Yields of Bacteria Containing plasmid
5 19 or plasmid 24:

To determine if the presence of the *ant*(4')-Ia gene coding sequence in a plasmid vector backbone would influence production of plasmid DNA, three fermentations of plasmid 24 were compared with two fermentations of plasmid 19. Each
10 plasmid vector is in *E. coli* strain DH10B, and the same fermentation and DNA purification protocols were performed for each strain.

Representative growth curves for the two bacterial strains are shown in Figure 16. The plasmid 24 strain grows
15 much more rapidly than the plasmid 19 strain, and reaches nearly twice the OD₆₀₀ after ten hours of fermentation. The plasmid DNA yields for each strain were also compared (Table 3). More plasmid 24 DNA was produced than plasmid 19, but the amounts are proportional to the cell yield. Thus, bacteria
20 containing plasmid 24 or plasmid 19 produce similar amounts of plasmid DNA, but because the plasmid 24 strain grows so much better, the yield of DNA from fermentation has improved substantially.

It is likely that the growth advantage seen with
25 plasmid 24 is due to the biochemical activities of the ANT(4')-IA enzyme when compared with those of the APH(3')-IA enzyme. The ATP used as a phosphate donor by APH(3')-IA is limited in concentration in growing cells. Given the ability of APH(3')-IA to phosphorylate a wide range of cellular substrates,
30 including kanamycin and water, bacteria harboring this enzyme to grow more slowly due to futile cycles of ATP generation followed by APH(3')-Ia mediated ATP breakdown.

ANT(4')-IA enzyme may have additional cellular activities beyond conferring drug resistance, including a
35 positive effect on cell growth. It is well known that cell growth is controlled by the levels of several global growth regulators, including cyclic AMP (cAMP), leucine and glutamine.

- 34 -

In particular, cAMP is a negative global growth regulator, in that high cellular levels of this metabolite are associated with low growth rate, while low cAMP levels are associated with a high growth rate. Since ANT(4')-IA enzyme acts by cleaving
5 nucleotides, cAMP may serve as a substrate for the enzyme.

To assess the cAMP phosphodiesterase activity in *E. coli* alone, and in *E. coli* with plasmids carrying either *aph(3')-Ia* or *ant(4')-Ia* an experiment was done. *E. coli* with the *ant(4')-Ia* gene possess 320-fold more cAMP
10 phosphodiesterase activity than *E. coli* alone, and 400-fold more activity than *E. coli* bearing *aph(3')-Ia*. Lower intracellular levels of cAMP may account for the improved cellular growth rate seen in *E. coli* bearing *ant(4')-Ia*. That is, the elevated cAMP phosphodiesterase activity seen in *E.*
15 *coli* that expresses ANT(4')-IA enzyme, may leads to lower levels of cAMP which could account for higher cellular growth.

The beneficial biochemical effects of the chimeric *ant(4')-Ia* gene could be conferred to host cells in either of two ways. The *ant(4')-Ia* gene could be supplied on a plasmid,
20 as in the case of plasmid 24. Alternatively, the *ant(4')-Ia* gene could be integrated into the chromosomal DNA of cells. Two examples follow. First, to generate a mammalian cell line with the *ant(4')-Ia* gene integrated into the chromosome, one would transfect cells with a plasmid containing *ant(4')-Ia*, and
25 select for cell clones stably resistant to neomycin (neomycin, but not kanamycin, is toxic to mammalian cells, and as shown previously, *ant(4')-Ia* confers resistance to neomycin). Second, an *E. coli* strain with the *ant(4')-Ia* gene integrated into the chromosome could be generated by homologous
30 recombination. In this case, one would insert the *ant(4')-Ia* gene into the center of 1-2 kb of cloned *E. coli* DNA, and use the resulting linear fragment to transform *E. coli* (C. Satishchandran, et al., 1991 *J. Bacteriol.* 172:4489-4496 incorporated herein). Kanamycin-resistant strains would be
35 selected for and analyzed molecularly to show that the desired recombination event occurred.

CONCLUSIONS

- 35 -

A hybrid kanamycin resistance gene which utilizes the *E. coli* *aph(3')*-Ia promoter and terminator to control expression of the *ant(4')*-Ia coding region is described. The first and second codons of the engineered gene have been
5 altered to ensure efficient expression of the gene. When the sensitivity spectrum of *E. coli* strains carrying *ant(4')*-Ia was compared with that of strains carrying *aph(3')*-Ia, *ant(4')*-Ia conferred resistance only to kanamycin, neomycin and tobramycin, while *aph(3')*-Ia conferred resistance to kanamycin,
10 neomycin, tobramycin, netilmicin, and gentamicin. Thus, the engineered gene has a more restricted range of activity and represents a significant safety improvement relative to clinical vectors which employ the *aph(3')*-Ia gene. The vector backbones with the *ant(4')*-Ia gene support good expression from
15 the eukaryotic promoter contained in the backbone. Finally, the presence of the *ant(4')*-Ia gene in the backbone is a manufacturing improvement, in that bacteria bearing plasmid 23-derived vectors grow significantly better and consequently produce more DNA.

Table 1. PRIMERS AND OLIGOMERS

PCR PRIMERS	SEQUENCE OF PRIMERS (5' TO 3')
MPV37	GGCCGGCCGGGGAAAGCCACGTTGTGTCTC (SEQ ID NO:5)
MPV38	AACACCCCTTGTATTACTGTTTATGTAAG (SEQ ID NO:6)
MPV39	GTGAATGGACCAATAATAATGACTAGAG (SEQ ID NO:7)
MPV40	CGCGCTCGTCGTATAACAGATGCG (SEQ ID NO:8)
MPV41	TCGGTCTTAACTGAAGCAGTTAAGC (SEQ ID NO:9)
MPV42	CGTTCAAATGGTATGCGTTTTGACAC (SEQ ID NO:10)
MPV43	CAGAATTGGTTAATTGGTTGTAACACTG (SEQ ID NO:11)
MPV44	ATTTAAATGGGGGCGCTGAGGTCTGCCTCG (SEQ ID NO:12)
MPV62	ATGAACGGACCAATAATAATGACTAGAGAAGAAAG (SEQ ID NO:13)
MPV63	CGGGATCCATTTAAATGGGGGCGCTGAGGTCTG (SEQ ID NO:14)
MPV64	GCTCTAGAGGCCGGCCGGGGAAAGCCACG (SEQ ID NO:15)
BRIDGE OLIGOMERS	
MPV45	CAGTAATACAAGGGGTGTTGTGAATGGACCAATAATAATG (SEQ ID NO:16)
MPV46	GTTATACGACGAGCGCGTCGGTCTTAACTGAAGCAG (SEQ ID NO:17)
MPV47	CGCATACCATTTTGAACGCAGAATTGGTTAATTGGTTG (SEQ ID NO:18)
MPV67	CAGTAATACAAGGGGTGTTATGAACGGACCAATAATAATG (SEQ ID NO:19)
MPV73	CACAACGTGGCTTTCCCCGGCCCATGACCAAATCCCTTAACGTGAG (SEQ ID NO:20)
MPV92	CAGGGGGAGGTGTGGGAGGTTTTTTAAATGGGGGCGCTGAGGTCTGCC (SEQ ID NO:21)

Table 2. Spectrum of Activity of ANT(4')-IA and APH(3')-IA
Against Aminoglycosides

Aminoglycoside	DH10B	DH10B/pBLUEkm ^{ant}	DH10B/pUC4K
kanamycin	1.0 S	32 R	32 R
neomycin	0.5 S	32 R	32 R
tobramycin	1.0 S	16 R	8 R
gentamicin	0.5 S	0.25 S	5 R
netilmicin	0.12 S	0.25 S	25 R
streptomycin	128 R	128 R	128 R
spectinomycin	4.0 S	4.0 S	4.0 S

Table 3. Yields of plasmid 19 and plasmid 24 DNA After
Fermentation

	plasmid 19	plasmid 24	24/19
Cells (g/l)	46	86	1.86
Plasmid DNA (mg/l)	13	22	1.69

- 38 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Pachuk, Catherine J.
 Samuel, Manoj
 Zurawski, John A.
 Satishchandran, C.

(ii) TITLE OF INVENTION: CHAIN REACTION CLONING

(iii) NUMBER OF SEQUENCES: 21

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Woodcock Washburn Kurtz Mackiewicz & Norris
 (B) STREET: One Liberty Place, 46th floor
 (C) CITY: Philadelphia
 (D) STATE: Pennsylvania
 (E) COUNTRY: USA
 (F) ZIP: 19103

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: WINDOWS
 (D) SOFTWARE: WordPerfect

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
 (B) FILING DATE:
 (C) CLASSIFICATION:

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/642,045
 (B) FILING DATE: 06-MAY-1996
 (C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: DeLuca, Mark
 (B) REGISTRATION NUMBER: 33,229
 (C) REFERENCE/DOCKET NUMBER: APOL-0294

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 215-568-3100
 (B) TELEFAX: 215-568-3439

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1200 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: both
 (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS
 (B) LOCATION: 131..892

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCTCTAGAGG CCGGCCGGGG AAAGCCACGT TGTGTCTCAA AATCTCTGAT GTTACATTGC 60

ACAAGATAAA AATATATCAT CATGAACAAT AAAACTGTCT GCTTACATAA ACAGTAATAC 120

AAGGGGTGTT ATG AAC GGA CCA ATA ATA ATG ACT AGA GAA GAA AGA ATG 169

Met Asn Gly Pro Ile Ile Met Thr Arg Glu Glu Arg Met

1

5

10

- 39 -

AAG ATT GTT CAT GAA ATT AAG GAA CGA ATA TTG GAT AAA TAT GGG GAT	217
Lys Ile Val His Glu Ile Lys Glu Arg Ile Leu Asp Lys Tyr Gly Asp	
15 20 25	
GAT GTT AAG GCT ATT GGT GTT TAT GGC TCT CTT GGT CGT CAG ACT GAT	265
Asp Val Lys Ala Ile Gly Val Tyr Gly Ser Leu Gly Arg Gln Thr Asp	
30 35 40 45	
GGG CCC TAT TCG GAT ATT GAG ATG ATG TGT GTC ATG TCA ACA GAG GAA	313
Gly Pro Tyr Ser Asp Ile Glu Met Met Cys Val Met Ser Thr Glu Glu	
50 55 60	
GCA GAG TTC AGC CAT GAA TGG ACA ACC GGT GAG TGG AAG GTG GAA GTG	361
Ala Glu Phe Ser His Glu Trp Thr Thr Gly Glu Trp Lys Val Glu Val	
65 70 75	
AAT TTT GAT AGC GAA GAG ATT CTA CTA GAT TAT GCA TCT CAG GTG GAA	409
Asn Phe Asp Ser Glu Glu Ile Leu Leu Asp Tyr Ala Ser Gln Val Glu	
80 85 90	
TCA GAT TGG CCG CTT ACA CAT GGT CAA TTT TTC TCT ATT TTG CCG ATT	457
Ser Asp Trp Pro Leu Thr His Gly Gln Phe Phe Ser Ile Leu Pro Ile	
95 100 105	
TAT GAT TCA GGT GGA TAC TTA GAG AAA GTG TAT CAA ACT GCT AAA TCG	505
Tyr Asp Ser Gly Gly Tyr Leu Glu Lys Val Tyr Gln Thr Ala Lys Ser	
110 115 120 125	
GTA GAA GCC CAA ACG TTC CAC GAT GCG ATT TGT GCC CTT ATC GTA GAA	553
Val Glu Ala Gln Thr Phe His Asp Ala Ile Cys Ala Leu Ile Val Glu	
130 135 140	
GAG CTG TTT GAA TAT GCA GGC AAA TGG CGT AAT ATT CGT GTG CAA GGA	601
Glu Leu Phe Glu Tyr Ala Gly Lys Trp Arg Asn Ile Arg Val Gln Gly	
145 150 155	
CCG ACA ACA TTT CTA CCA TCC TTG ACT GTA CAG GTA GCA ATG GCA GGT	649
Pro Thr Thr Phe Leu Pro Ser Leu Thr Val Gln Val Ala Met Ala Gly	
160 165 170	
GCC ATG TTG ATT GGT CTG CAT CAT CGC ATC TGT TAT ACG ACG AGC GCG	697
Ala Met Leu Ile Gly Leu His His Arg Ile Cys Tyr Thr Thr Ser Ala	
175 180 185	
TCG GTC TTA ACT GAA GCA GTT AAG CAA TCA GAT CTT CCT TCA GGT TAT	745
Ser Val Leu Thr Glu Ala Val Lys Gln Ser Asp Leu Pro Ser Gly Tyr	
190 195 200 205	
GAC CAT CTG TGC CAG TTC GTA ATG TCT GGT CAA CTT TCC GAC TCT GAG	793
Asp His Leu Cys Gln Phe Val Met Ser Gly Gln Leu Ser Asp Ser Glu	
210 215 220	
AAA CTT CTG GAA TCG CTA GAG AAT TTC TGG AAT GGG ATT CAG GAG TGG	841
Lys Leu Leu Glu Ser Leu Glu Asn Phe Trp Asn Gly Ile Gln Glu Trp	
225 230 235	
ACA GAA CGA CAC GGA TAT ATA GTG GAT GTG TCA AAA CGC ATA CCA TTT	889
Thr Glu Arg His Gly Tyr Ile Val Asp Val Ser Lys Arg Ile Pro Phe	
240 245 250	
TGA ACGCAGAATT GGTAAATTGG TTGTAACACT GGCAGAGCAT TACGCTGACT	942
*	
TGACGGGACG GCGGCTTTGT TGAATAAATC GAACTTTTGC TGAGTTGAAG GATCAGATCA	1002
CGCATCTTCC CGACAACGCA GACCGTTCCG TGGCAAAGCA AAAGTTCAAA ATCACCAACT	1062

- 40 -

GGTCCACCTA CAACAAAGCT CTCATCAACC GTGGCTCCCT CACTTTCTGG CTGGATGATG 1122
 GGGCGATTCA GGCCTGGTAT GAGTCAGCAA CACCTTCTTC ACGAGGCAGA CCTCAGCGCC 1182
 CCCATT TAAA TGGATCCG 1200

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 254 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Asn Gly Pro Ile Ile Met Thr Arg Glu Glu Arg Met Lys Ile Val
 1 5 10 15
 His Glu Ile Lys Glu Arg Ile Leu Asp Lys Tyr Gly Asp Asp Val Lys
 20 25 30
 Ala Ile Gly Val Tyr Gly Ser Leu Gly Arg Gln Thr Asp Gly Pro Tyr
 35 40 45
 Ser Asp Ile Glu Met Met Cys Val Met Ser Thr Glu Glu Ala Glu Phe
 50 55 60
 Ser His Glu Trp Thr Thr Gly Glu Trp Lys Val Glu Val Asn Phe Asp
 65 70 75 80
 Ser Glu Glu Ile Leu Leu Asp Tyr Ala Ser Gln Val Glu Ser Asp Trp
 85 90 95
 Pro Leu Thr His Gly Gln Phe Phe Ser Ile Leu Pro Ile Tyr Asp Ser
 100 105 110
 Gly Gly Tyr Leu Glu Lys Val Tyr Gln Thr Ala Lys Ser Val Glu Ala
 115 120 125
 Gln Thr Phe His Asp Ala Ile Cys Ala Leu Ile Val Glu Glu Leu Phe
 130 135 140
 Glu Tyr Ala Gly Lys Trp Arg Asn Ile Arg Val Gln Gly Pro Thr Thr
 145 150 155 160
 Phe Leu Pro Ser Leu Thr Val Gln Val Ala Met Ala Gly Ala Met Leu
 165 170 175
 Ile Gly Leu His His Arg Ile Cys Tyr Thr Thr Ser Ala Ser Val Leu
 180 185 190
 Thr Glu Ala Val Lys Gln Ser Asp Leu Pro Ser Gly Tyr Asp His Leu
 195 200 205
 Cys Gln Phe Val Met Ser Gly Gln Leu Ser Asp Ser Glu Lys Leu Leu
 210 215 220
 Glu Ser Leu Glu Asn Phe Trp Asn Gly Ile Gln Glu Trp Thr Glu Arg
 225 230 235 240
 His Gly Tyr Ile Val Asp Val Ser Lys Arg Ile Pro Phe *

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- 41 -

- (A) LENGTH: 9 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both
- (ii) MOLECULE TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTGAATGGA 9

- (2) INFORMATION FOR SEQ ID NO:4:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 9 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: both
 - (ii) MOLECULE TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATGAACGGA 9

- (2) INFORMATION FOR SEQ ID NO:5:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGCCGGCCGG GGAAAGCCAC GTTGTGTCTC 30

- (2) INFORMATION FOR SEQ ID NO:6:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AACACCCCTT GTATTACTGT TTATGTAAG 29

- (2) INFORMATION FOR SEQ ID NO:7:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTGAATGGAC CAATAATAAT GACTAGAG 28

- (2) INFORMATION FOR SEQ ID NO:8:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

- 42 -

CGCGCTCGTC GTATAACAGA TGCG 24

- (2) INFORMATION FOR SEQ ID NO:9:
(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 25 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TCGGTCTTAA CTGAAGCAGT TAAGC 25

- (2) INFORMATION FOR SEQ ID NO:10:
(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CGTTCAAAAT GGTATGCGTT TTGACAC 27

- (2) INFORMATION FOR SEQ ID NO:11:
(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 28 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CAGAATTGGT TAATTGGTTG TAACACTG 28

- (2) INFORMATION FOR SEQ ID NO:12:
(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ATTTAAATGG GGGCGCTGAG GTCTGCCTCG 30

- (2) INFORMATION FOR SEQ ID NO:13:
(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 35 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ATGAACGGAC CAATAATAAT GACTAGAGAA GAAAG 35

- (2) INFORMATION FOR SEQ ID NO:14:
(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 33 base pairs
 (B) TYPE: nucleic acid

- 43 -

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CGGGATCCAT TTAAATGGGG GCGCTGAGGT CTG 33

- (2) INFORMATION FOR SEQ ID NO:15:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GCTCTAGAGG CCGGCCGGGG AAAGCCACG 29

- (2) INFORMATION FOR SEQ ID NO:16:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 40 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CAGTAATACA AGGGGTGTTG TGAATGGACC AATAATAATG 40

- (2) INFORMATION FOR SEQ ID NO:17:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 36 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GTTATACGAC GAGCGCGTCG GTCTTAACTG AAGCAG 36

- (2) INFORMATION FOR SEQ ID NO:18:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 38 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CGCATACCAT TTTGAACGCA GAATTGGTTA ATTGGTTG 38

- (2) INFORMATION FOR SEQ ID NO:19:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 40 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CAGTAATACA AGGGGTGTTA TGAACGGACC AATAATAATG 40

- 44 -

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 47 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CACAAACGTGG CTTTCCCCGG CCCATGACCA AAATCCCTTA ACGTGAG 47

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 48 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CAGGGGGAGG TGTGGGAGGT TTTTAAATG GGGGCGCTGA GGTCTGCC 48

- 45 -

CLAIMS

1. A method of synthesizing a composite nucleic acid molecule by ligating a first nucleic acid molecule to a second nucleic acid molecule, the method comprising the steps of:

5 a) maintaining a first nucleic acid molecule and a second nucleic acid molecule at a denaturing temperature;

b) forming a mixture comprising said first nucleic acid molecule and said second nucleic acid molecule with a bridging oligonucleotide and a thermostable ligase, wherein
10 said bridging oligonucleotide comprises:

i) a 10-40 nucleotide sequence at its 5' end which is complementary to a nucleotide sequence on the 3' end of a strand of said first nucleic acid molecule,

ii) a 10-40 nucleotide sequence at its 3' end
15 which is complementary to a nucleotide sequence on the 5' end of a strand of said second nucleic acid molecule,

wherein at annealing temperature, said bridging oligonucleotide hybridizes to sequences on the sense strand of both the first and second nucleic acid molecule or on the
20 antisense strand of both the first and second nucleic acid molecule;

c) maintaining said mixture at an annealing temperature, wherein said bridging oligonucleotide hybridizes to sequences on of both the first and second nucleic acid
25 molecule and said thermostable ligase ligates either said 3' end of said first nucleic acid molecule to said 5' end of said second nucleic acid molecule or said 5' end of said first nucleic acid molecule to said 3' end of said second nucleic acid molecule to form a first strand of said composite nucleic
30 acid molecule hybridized to a bridging oligonucleotide;

d) maintaining said mixture at a denaturing temperature, wherein said bridging oligonucleotide dissociates from said first strand of said composite nucleic acid molecule;

e) maintaining said mixture at an annealing
35 temperature, wherein said first strand of said composite nucleic acid molecule hybridizes to complementary strands of said first nucleic acid molecules and said second nucleic acid

- 46 -

molecule, wherein said thermostable ligase ligates the end of said complementary strand of said first nucleic acid molecules to the end of said complementary strand of second nucleic acid molecule to form a second strand of said composite nucleic acid molecule.
5 molecule.

2. The method of claim 1 wherein steps c), d) and e) are repeated sequentially 2-50 times.

3. The method of claim 1 wherein the concentration of thermostable ligase in said mixture is 1-20 units/100ul
10 reaction.

4. The method of claim 3 wherein the concentration of thermostable ligase in said mixture is 5 units/100ul reaction.

5. The method of claim 1 wherein the amount of bridging
15 oligonucleotide provided in said mixture ranges from 1000 fold less to 1000 fold more relative to the amount of DNA present for ligation.

6. The method of claim 1 wherein the ratio of the amount of bridging oligonucleotide provided in said mixture to the
20 amount of DNA present for ligation is 1 to 1.

7. The method of claim 1 wherein the amount of DNA provided in said mixture ranges from .1 ng to 100 ug DNA.

8. A method of ligating a plurality of nucleic acid molecules comprising the steps of the method of claim 1
25 wherein said mixture comprises a plurality of nucleic acid molecules and a plurality of bridging oligonucleotides.

9. The method of claim 8 wherein said plurality of nucleic acid molecules consists of a vector and an insert.

10. The method of claim 8 wherein said plurality of

- 47 -

nucleic acid molecules comprise a plurality of polymerase chain reaction products.

11. The method of claim 10 wherein said plurality of nucleic acid molecules comprise a plurality of polymerase chain
5 reaction products produced using a non-strand displacing heat stable DNA polymerase.

Figure 1 continued

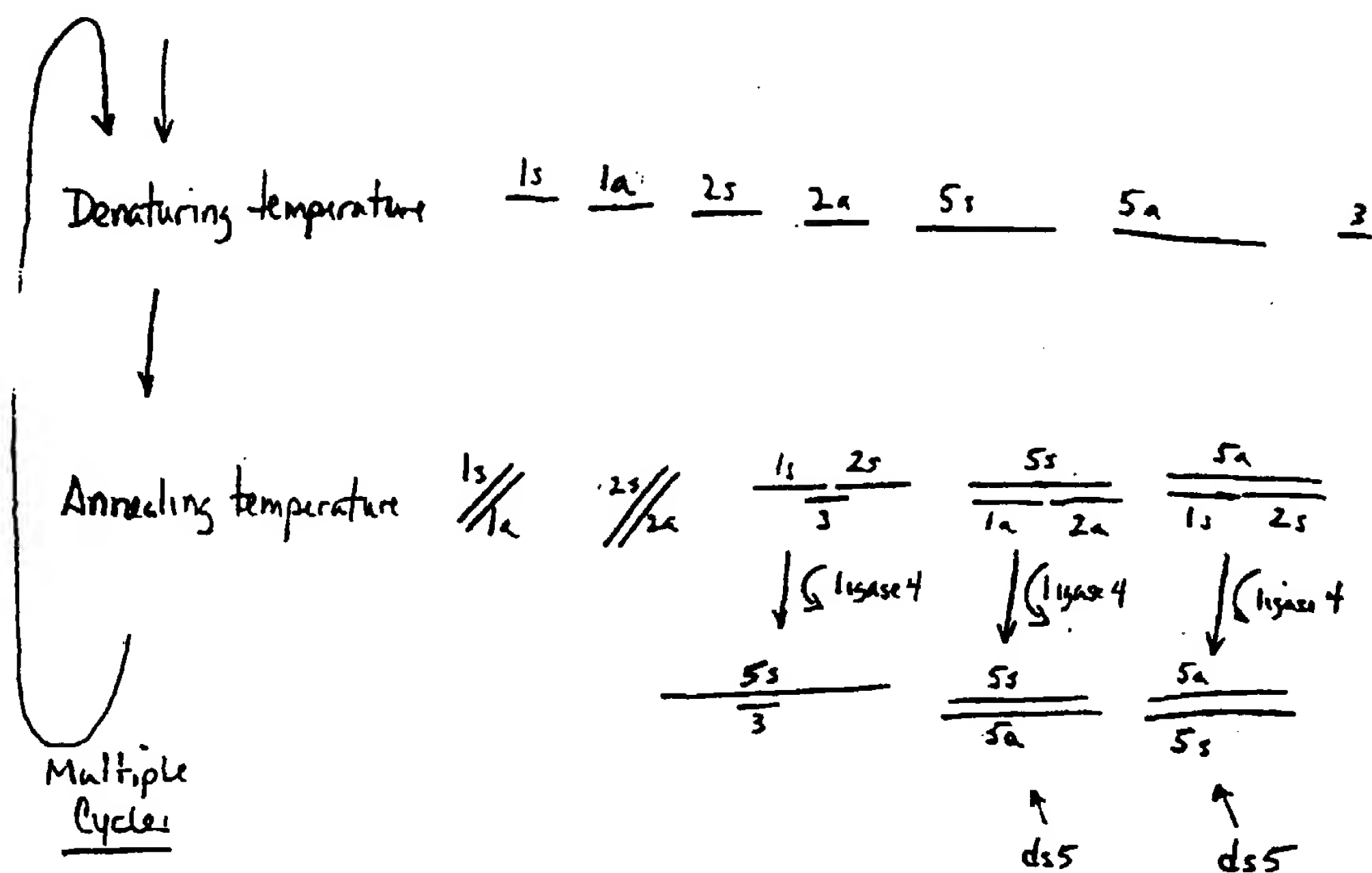


Figure 2

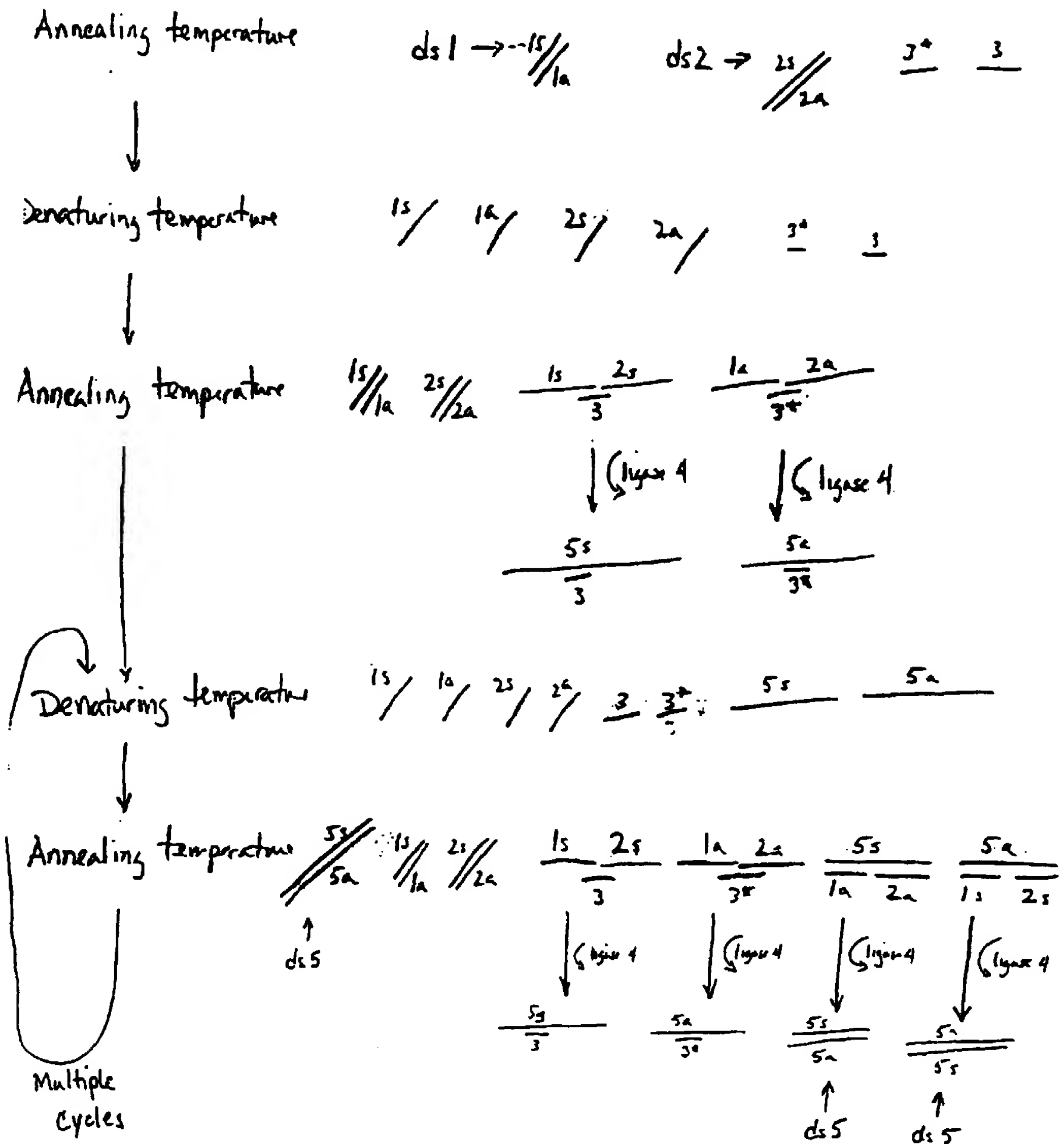


Figure 3



PCR
amplification

Denaturing
temperature

13s / 13'a / 14s / 14'a / 15s / 15'a / 16s / 16'a



Annealing
temperature

13s 13'a	14s 14'a	15s 15'a	16s 16'a
↑	↑	↑	↑
17	18	19	20

Figure 4

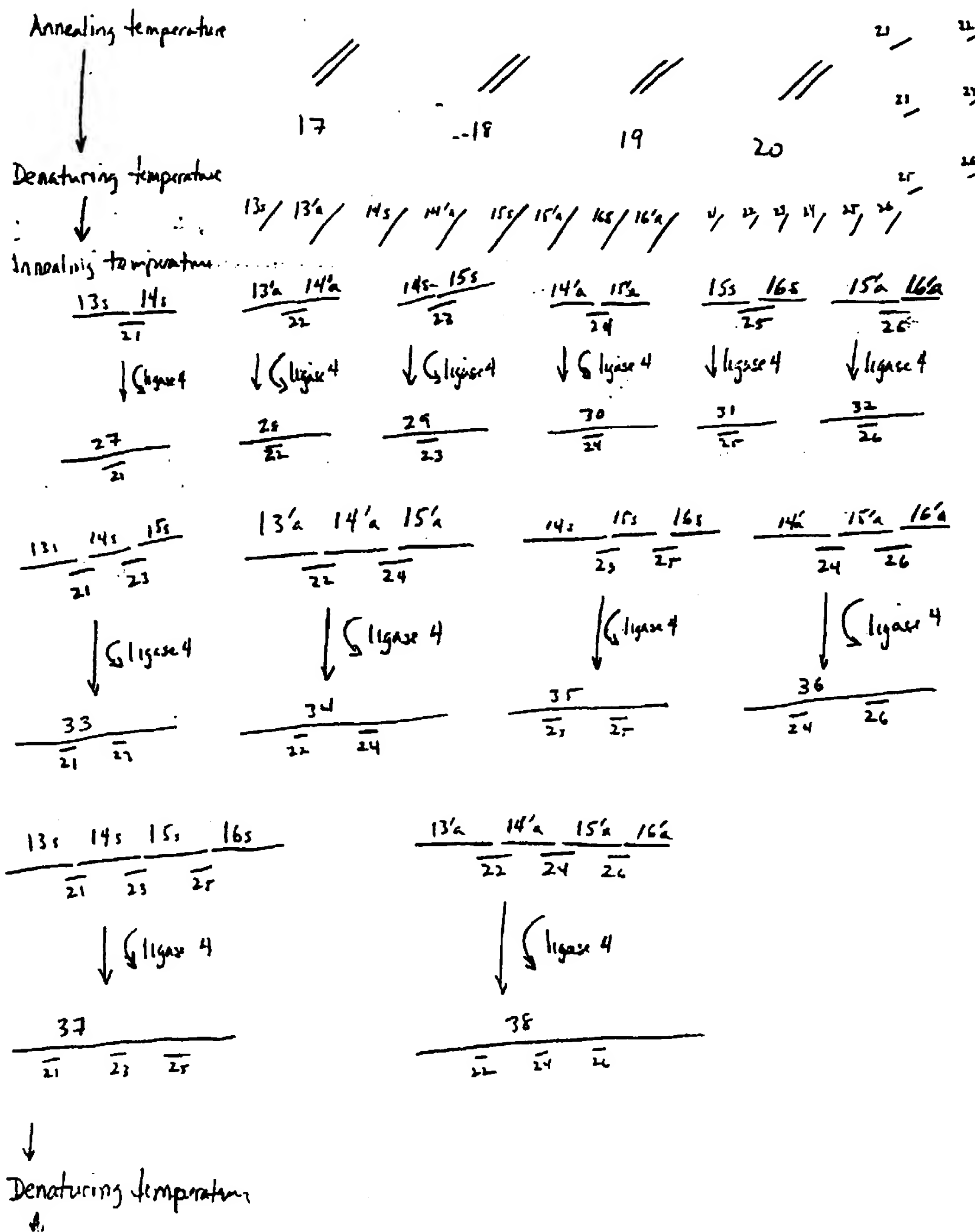


FIGURE 4 Continued

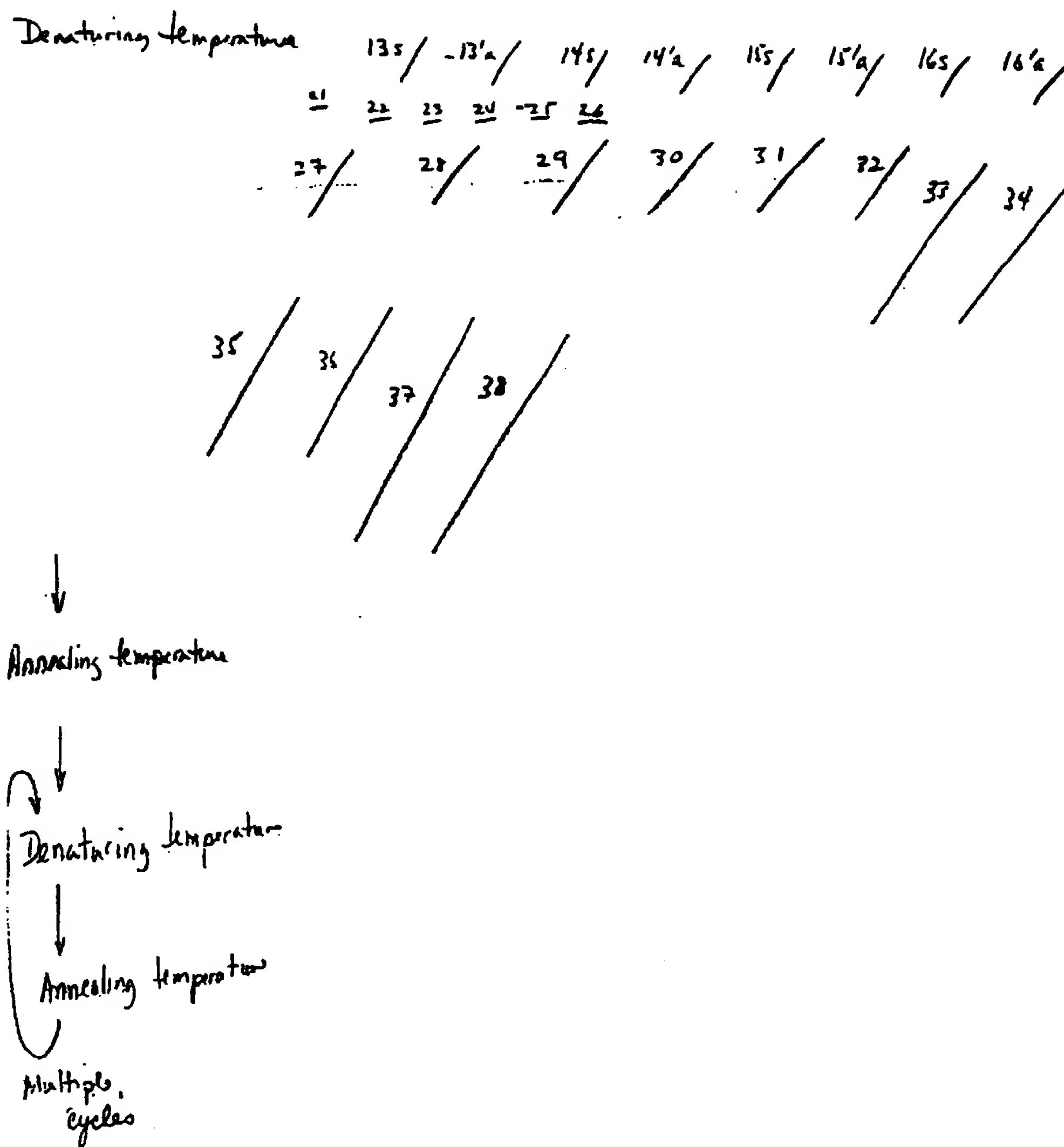
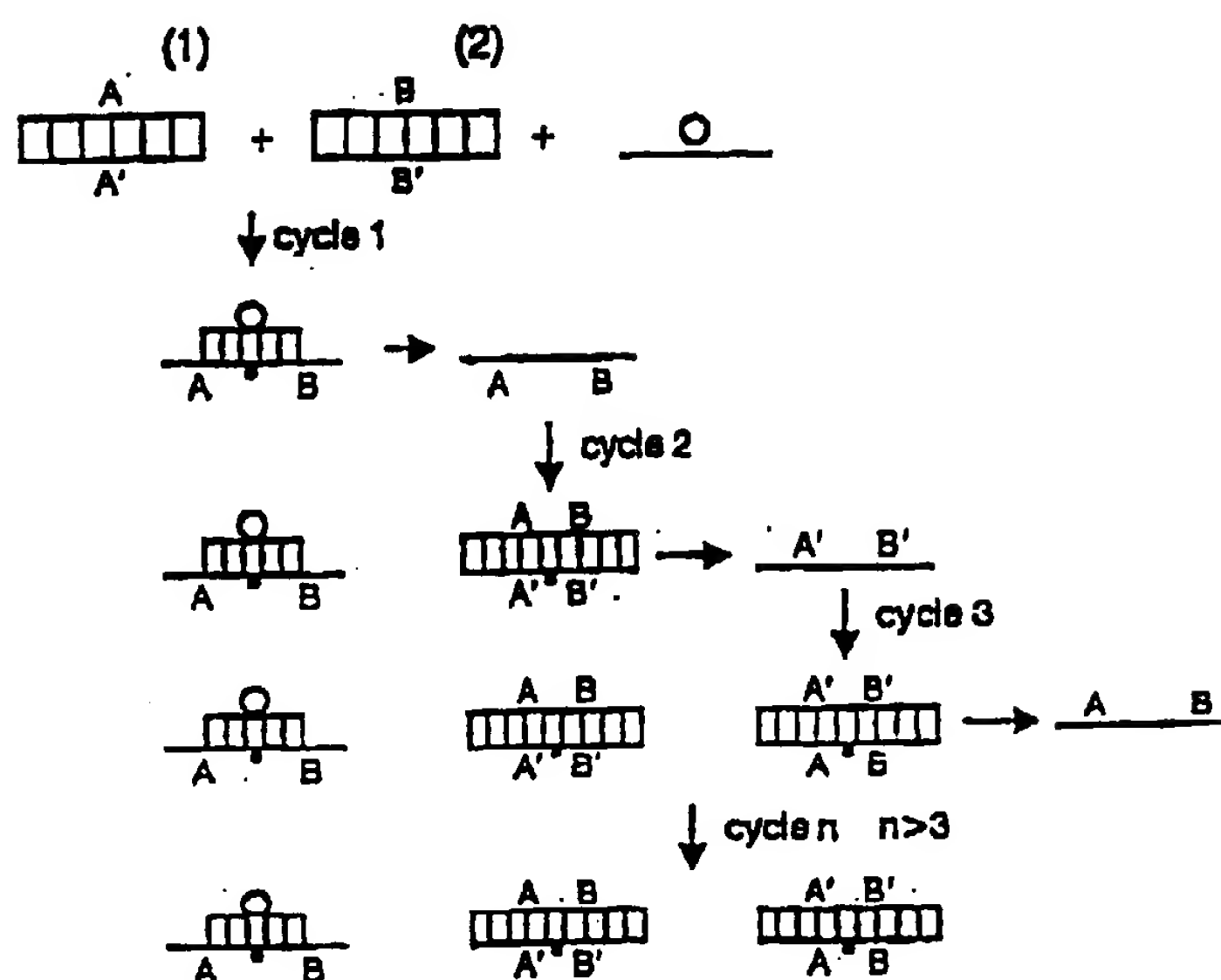
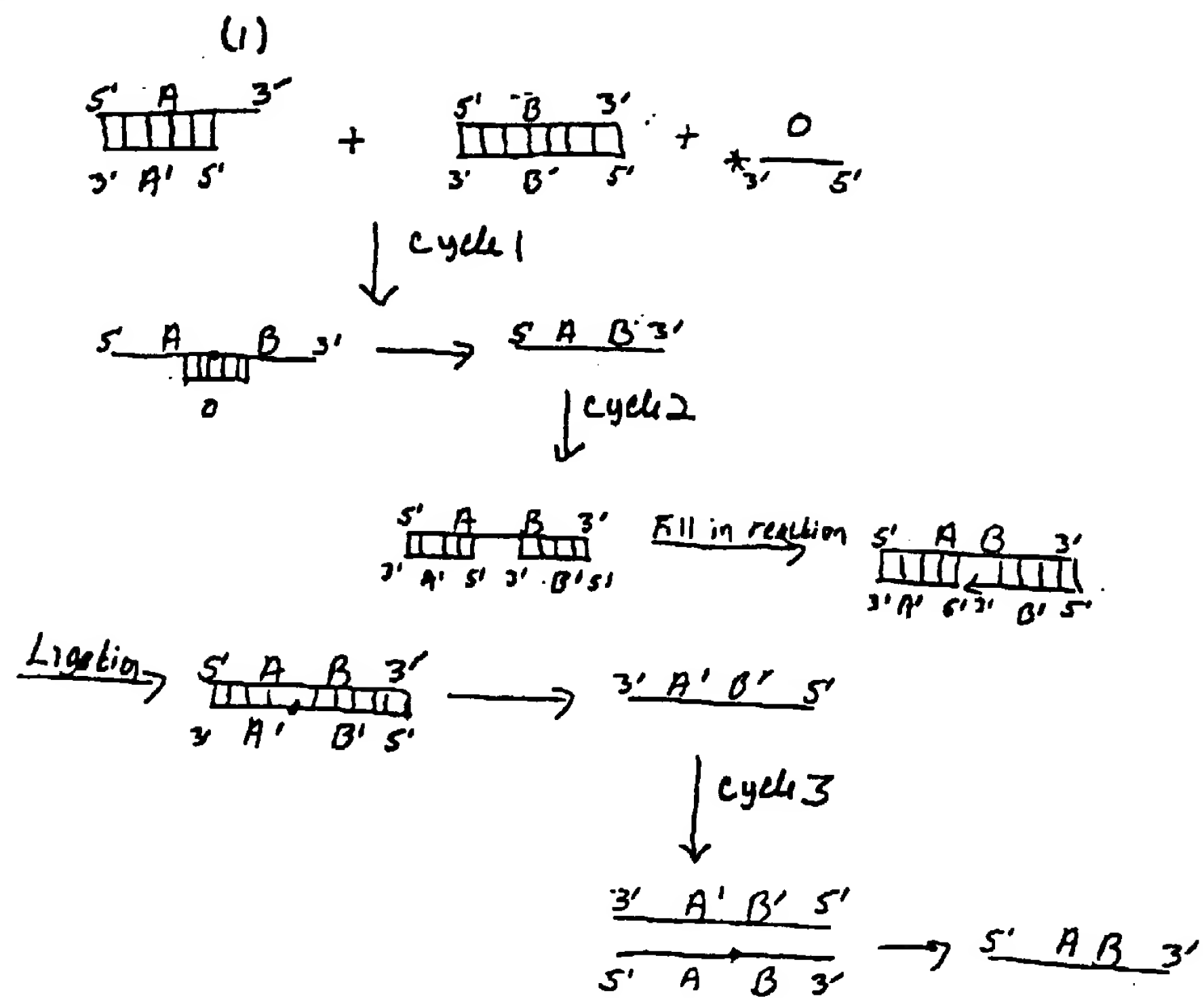


Fig 5A



8/19

Figure 5B.



9/19

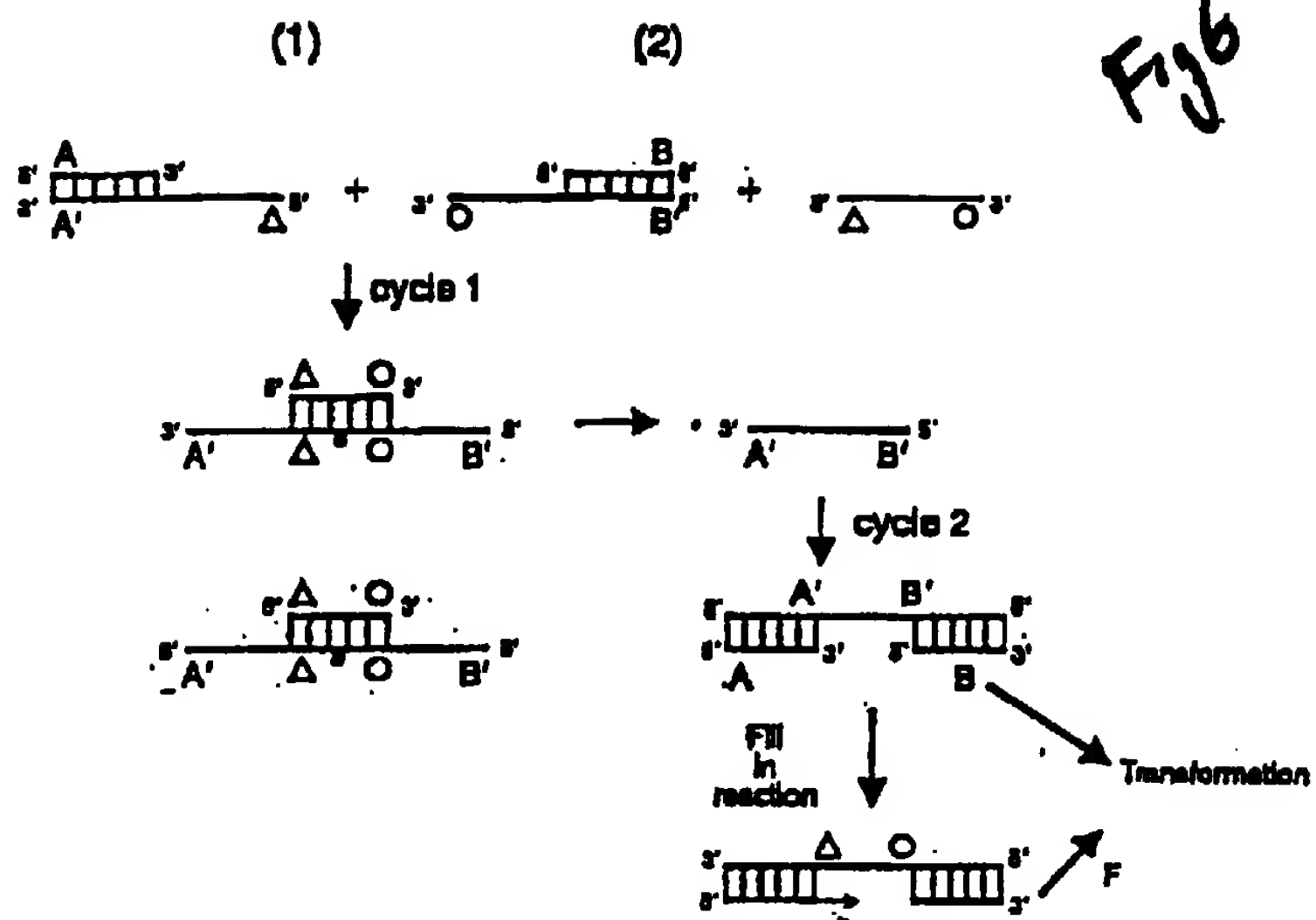


FIGURE 7A
Site Directed Deletion
of Sequences

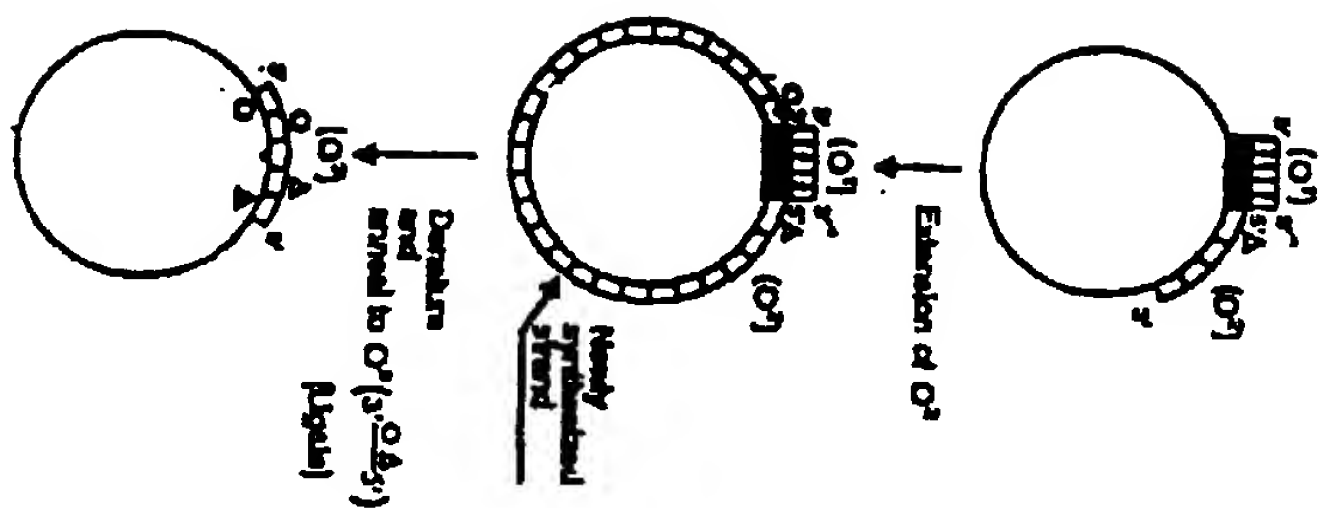


FIGURE 7B
Site Directed Insertion
of Sequences

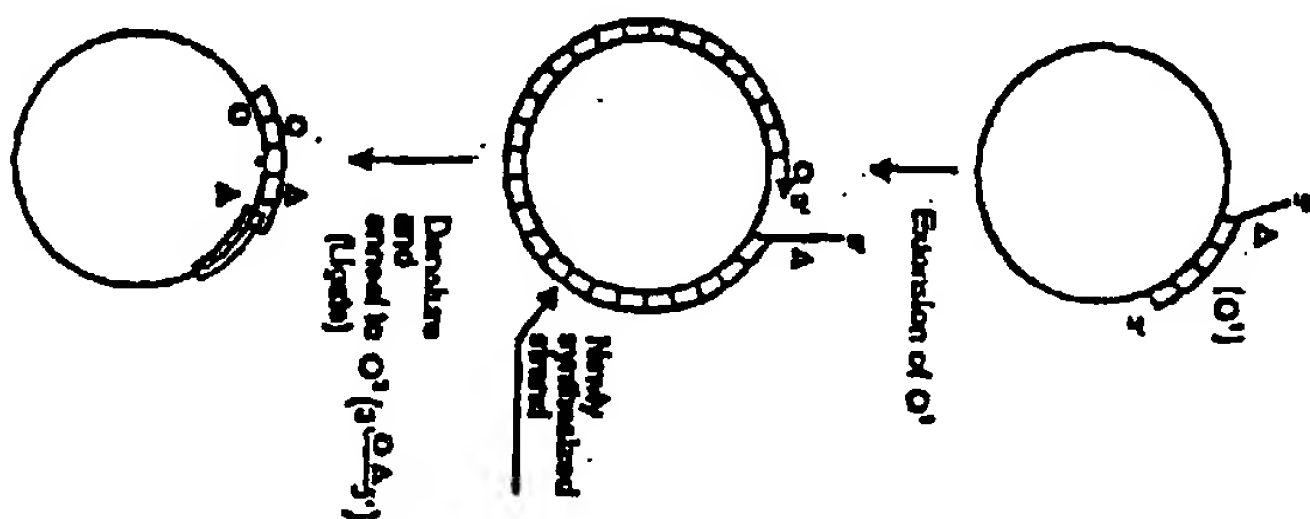


FIGURE 7C

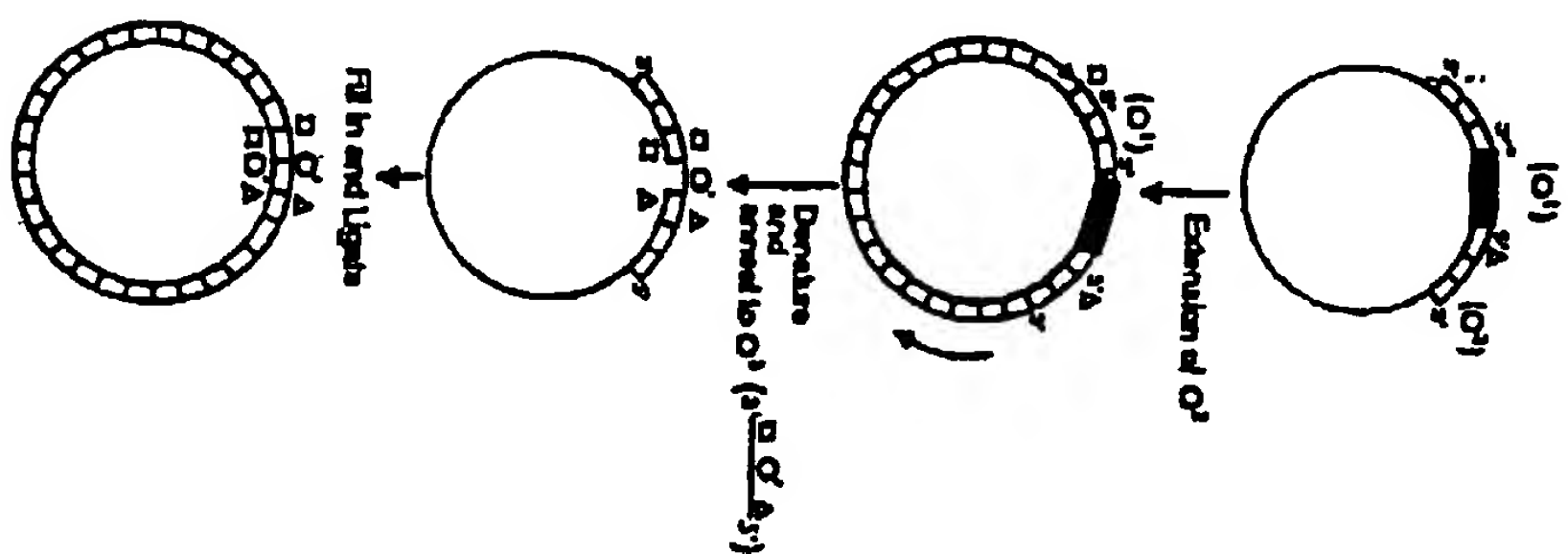
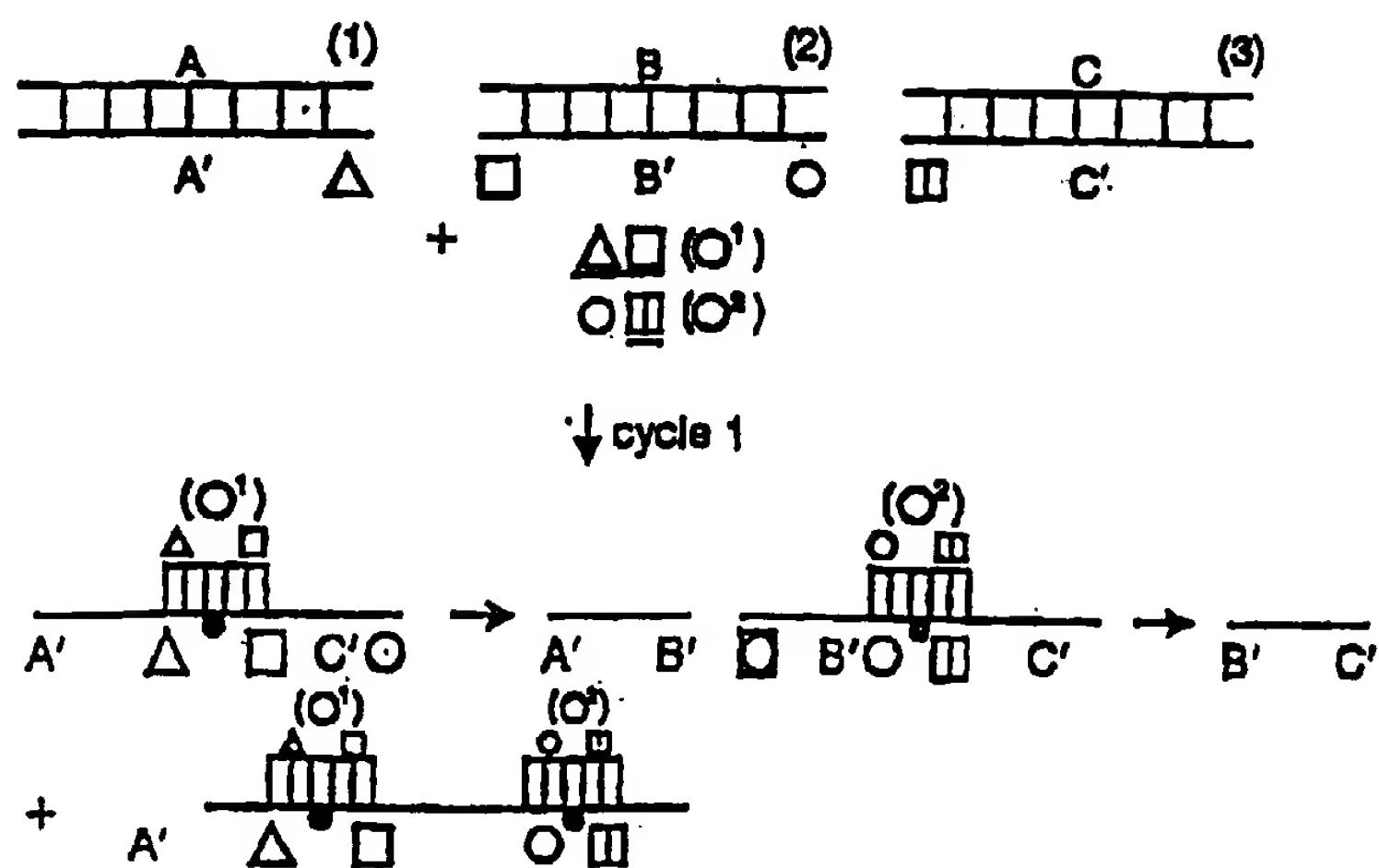


Fig 8



12/19

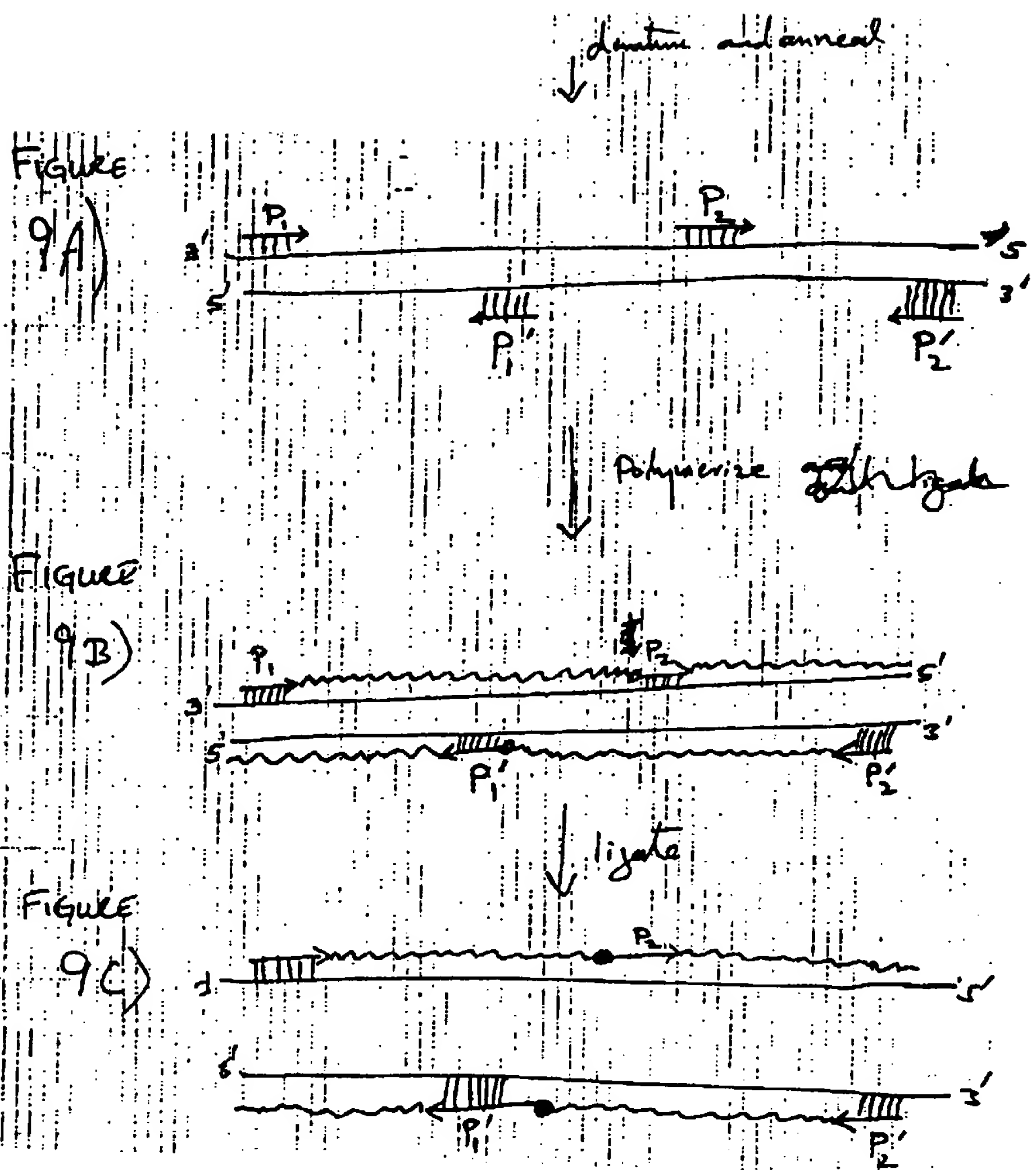


FIGURE 10A

PCR

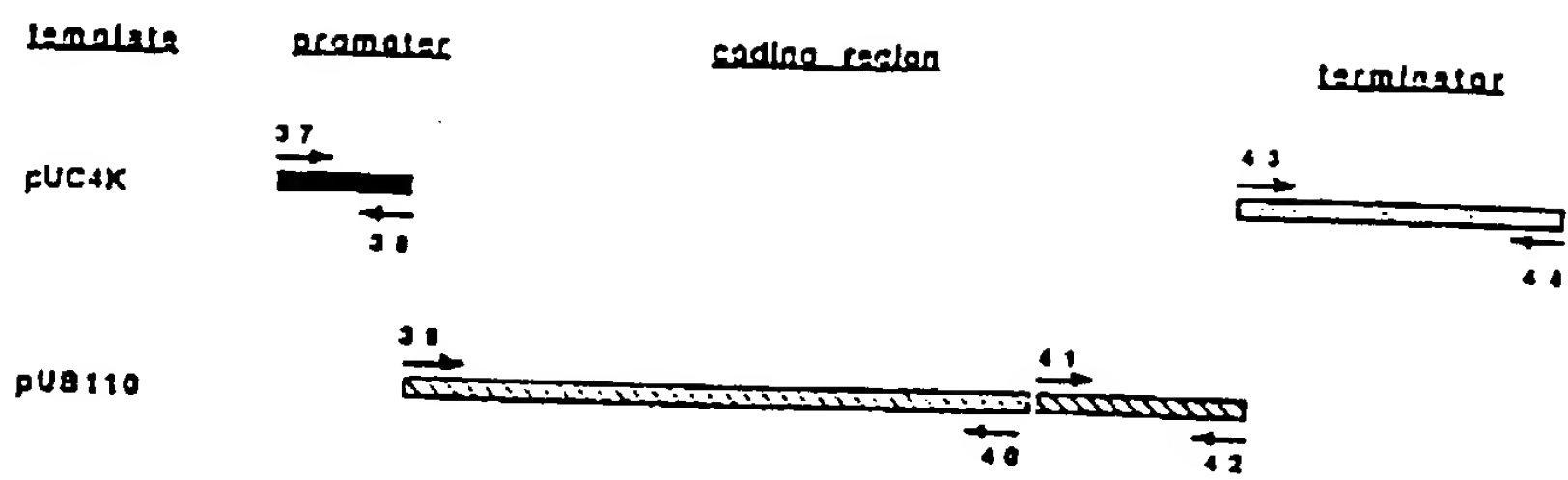


FIGURE 10B

CRC

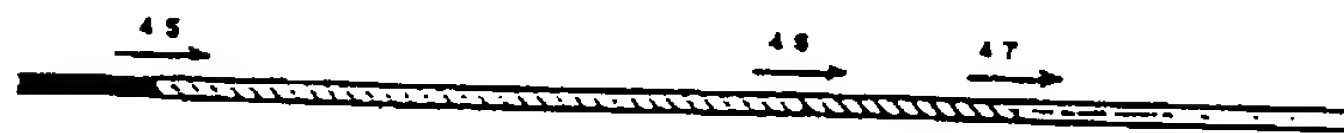


FIGURE 11

CAGTAATACAAGGGGTGTT | GTG AAT GGA CCA ATA A...
 M N G P I
 ATG GAC CAA TAA...
 M D Q •

FIGURE 12A

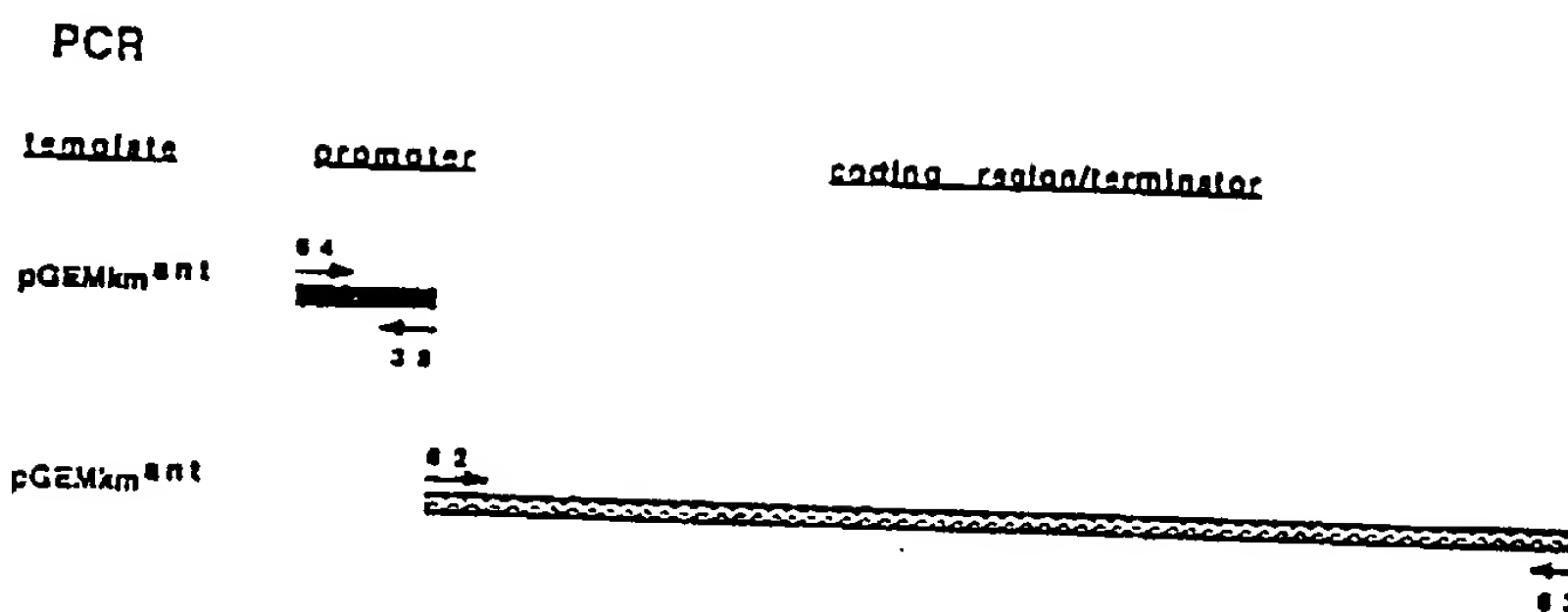


FIGURE 12B

CRC



FIGURE 13

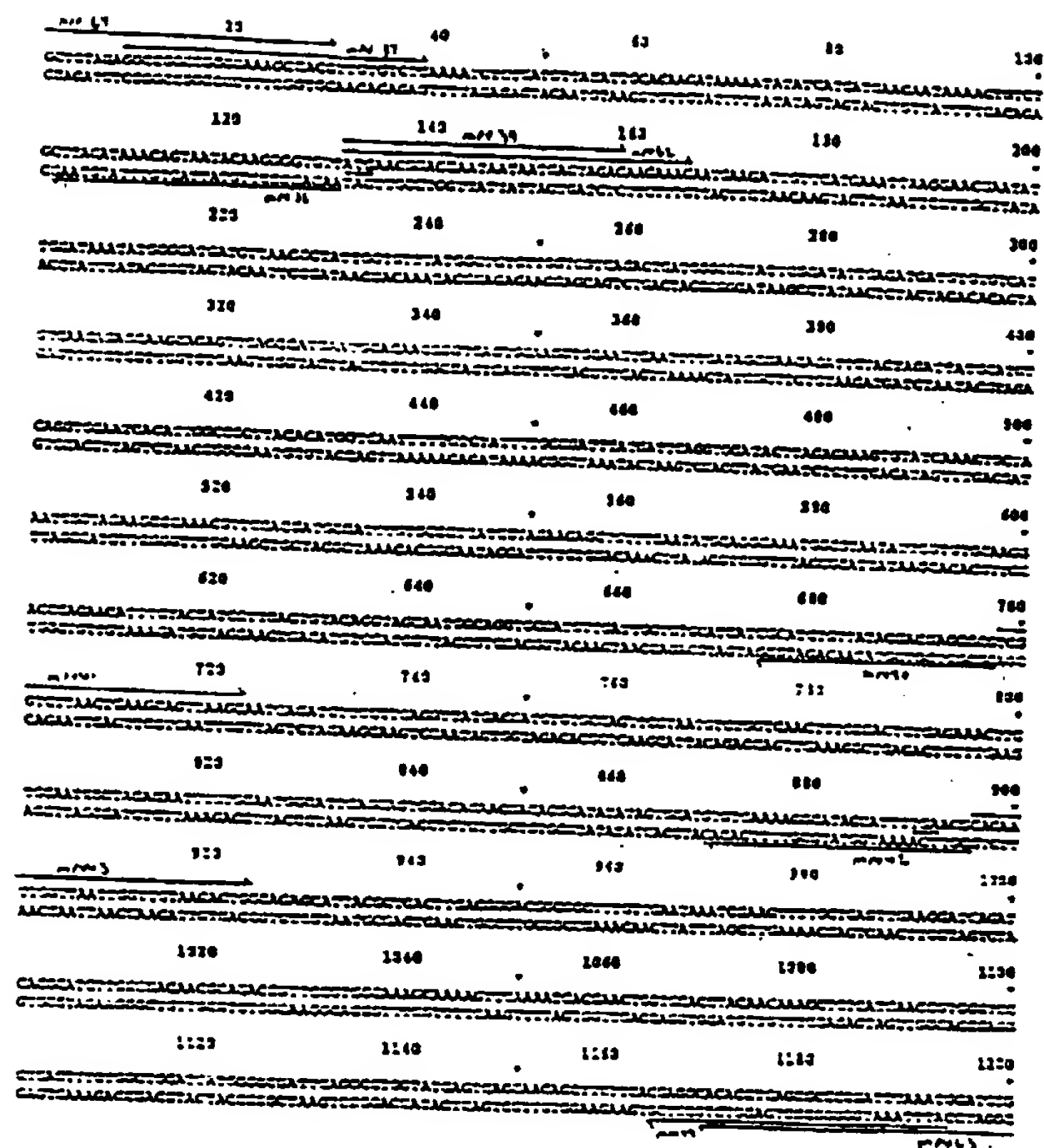


FIGURE 14

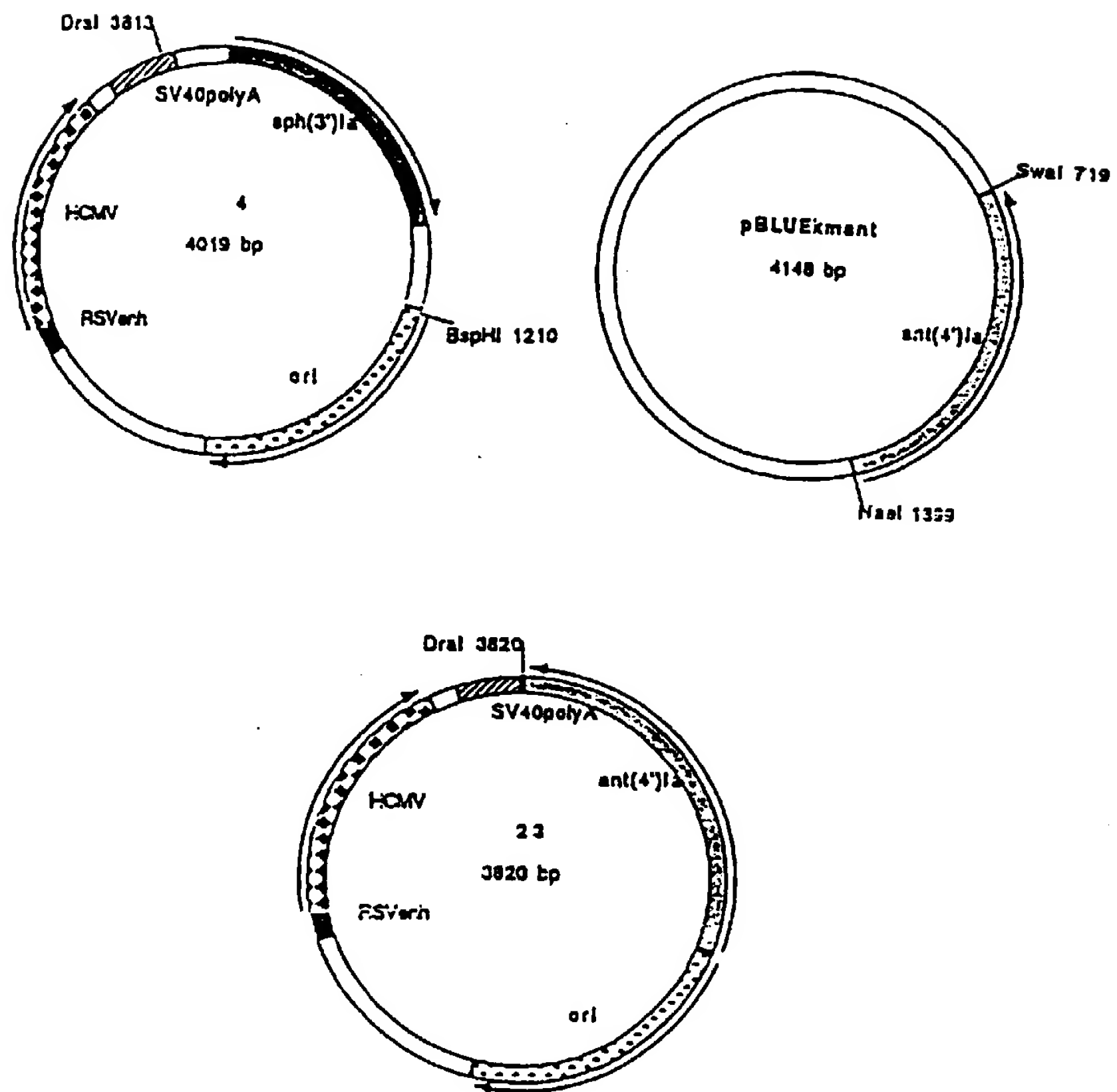


FIGURE 15A

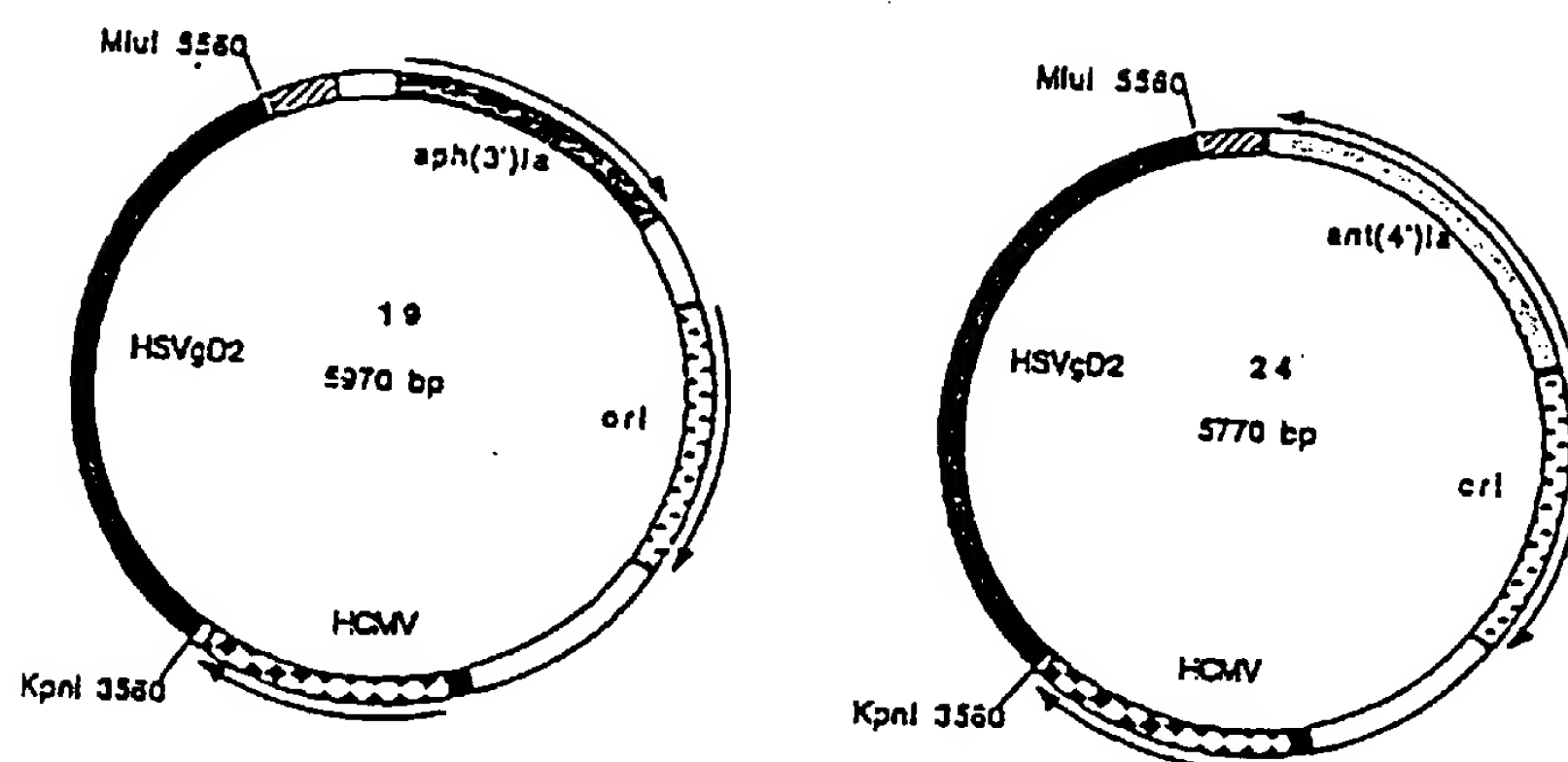
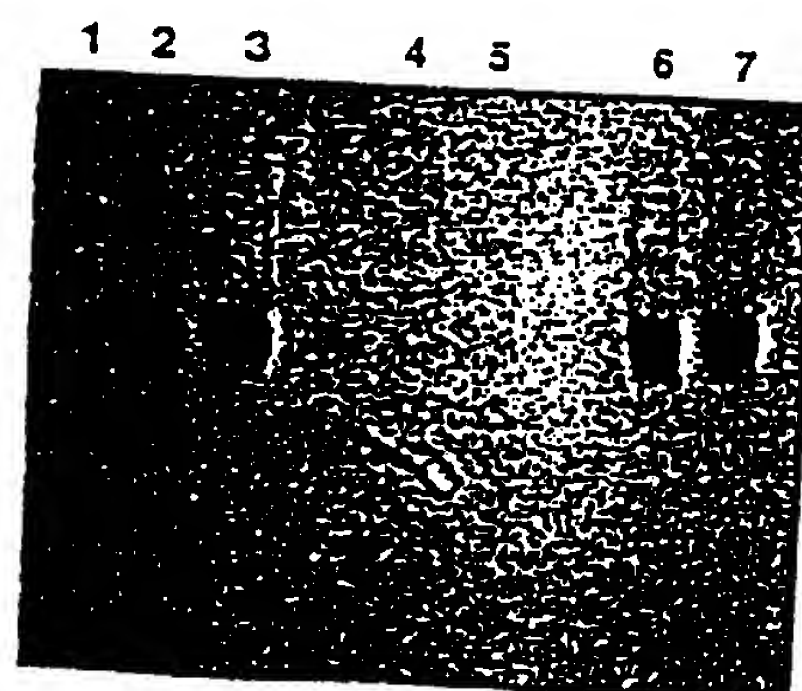
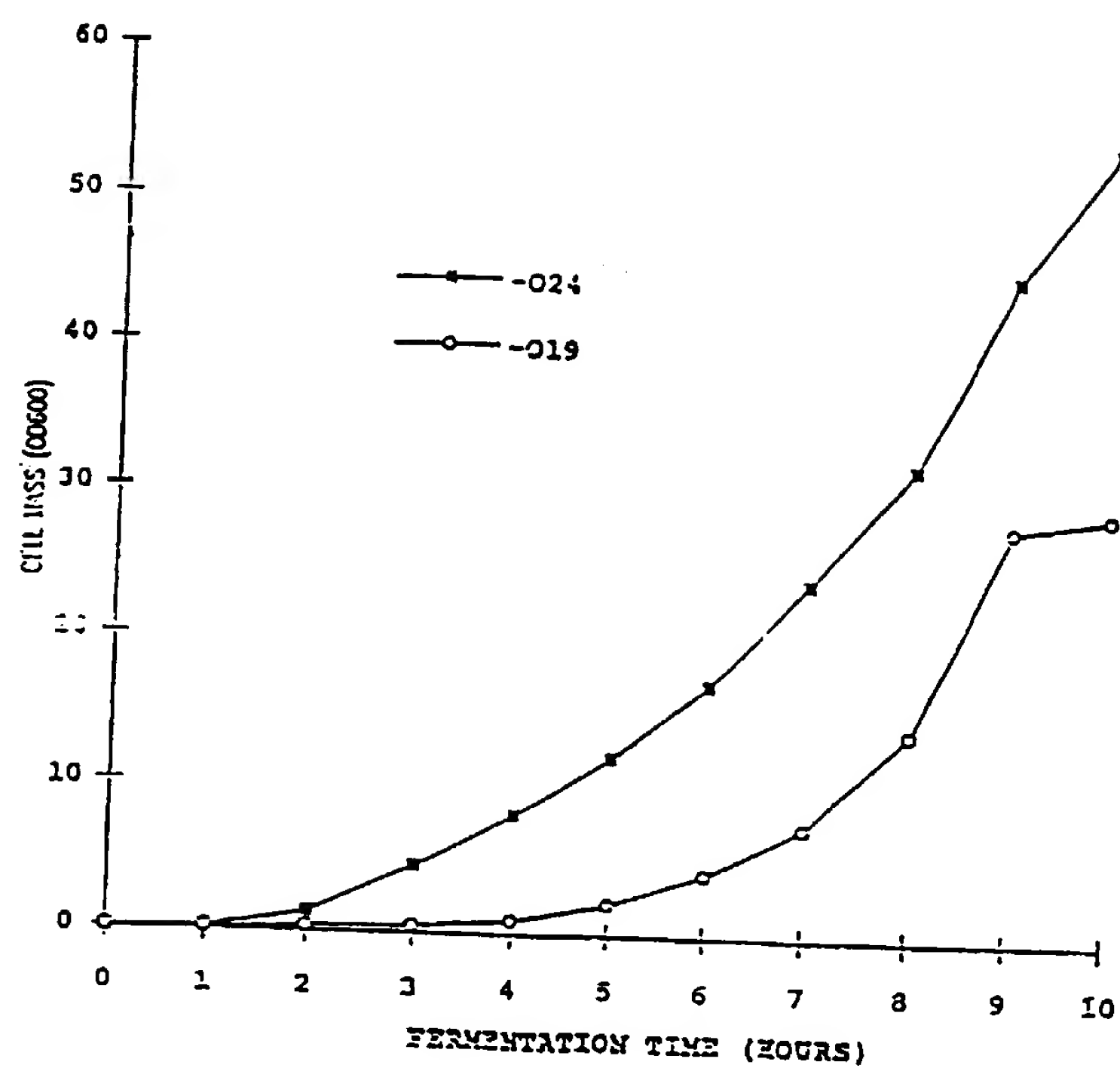


FIGURE 15B



19/19

FIGURE 16



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/07698

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 15/64; C12P 19/34

US CL : 435/91.2, 91.4, 91.41, 91.52

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/91.2, 91.4, 91.41, 91.52

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, EMBASE, SCISEARCH, CAPLUS, WPIDS
search terms: ligase#, ligation, thermostable, chain reaction, clon###,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	PACHUK et al. Chain reaction cloning: A novel cloning strategy. FASEB Journal. June 1996, Vol. 10, No. 6, page A1127, abstract No. 740.	1-11
A	ROUWENDAL et al. Simultaneous mutagenesis of multiple sites: Application of the ligase chain reaction using PCR products instead of oligonucleotides. BioTechniques. 1993, Vol. 15, No. 1, pages 69-71, 73-75, and 77.	1-11
A	DENG et al. Simultaneous randomization of antibody CDRs by a synthetic ligase chain reaction strategy. Nucleic Acids Research. 1993, Vol. 21, No. 18, pages 4418-4419.	1-11

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* T	Inter document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A		document defining the general state of the art which is not considered to be of particular relevance
* E		earlier document published on or after the international filing date
* L		document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
* O		document referring to an oral disclosure, use, exhibition or other means
* P		document published prior to the international filing date but later than the priority date claimed
	* X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
	* Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
	* &	document member of the same patent family

Date of the actual completion of the international search

14 JULY 1997

Date of mailing of the international search report

21 AUG 1997

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

SCOTT D. PRIEBE

Telephone No. (703) 308-0196

21/21

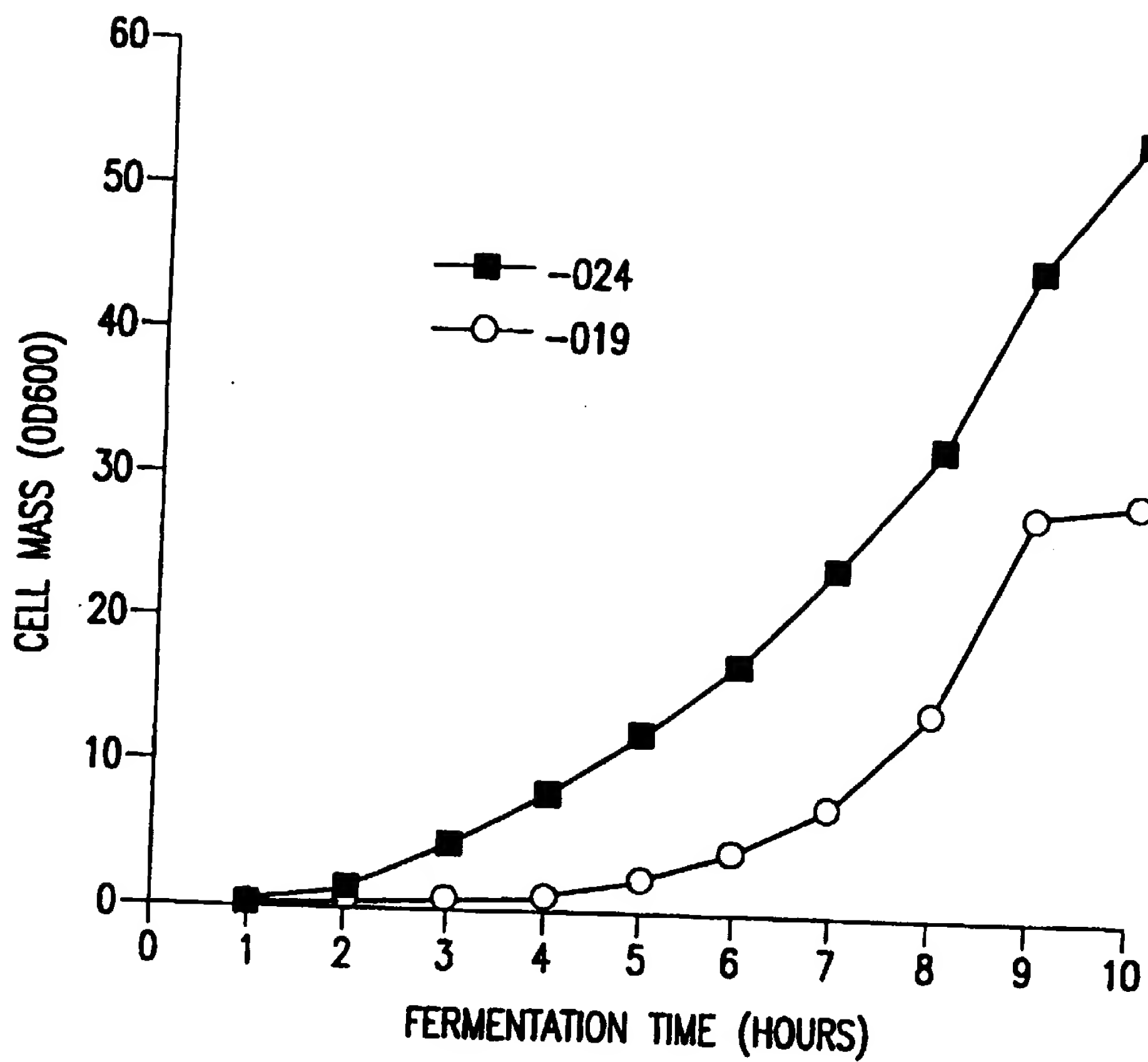


FIG.16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/07698

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/64; C12P 19/34
US CL :435/91.2, 91.4, 91.41, 91.52

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/91.2, 91.4, 91.41, 91.52

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, EMBASE, SCISEARCH, CAPLUS, WPIDS
search terms: ligase#, ligation, thermostable, chain reaction, clon###

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	PACHUK et al. Chain reaction cloning: A novel cloning strategy. FASEB Journal. June 1996, Vol. 10, No. 6, page A1127, abstract No. 740.	1-11
A	ROUWENDAL et al. Simultaneous mutagenesis of multiple sites: Application of the ligase chain reaction using PCR products instead of oligonucleotides. BioTechniques. 1993, Vol. 15, No. 1, pages 69-71, 73-75, and 77.	1-11
A	DENG et al. Simultaneous randomization of antibody CDRs by a synthetic ligase chain reaction strategy. Nucleic Acids Research. 1993, Vol. 21, No. 18, pages 4418-4419.	1-11

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"A" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search
14 JULY 1997

Date of mailing of the international search report

21 AUG 1997

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Authorized officer

SCOTT D. PRIEBE

Facsimile No. (703) 305-3230

Telephone No. (703) 308-0196